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# THE JOURNAL OF PHYSIOLOGY

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BY

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## THE COMPENSATORY MECHANISM OF THE SPLANCHNIC CIRCULATION DURING CHANGES OF POSTURE

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THE parts played by the splanchnic vascular area and the respiratory pump in compensating for the effects of gravity on the circulation were first emphasized by L. Hill [1895].

Subsequent work, which in general confirms Hill's findings, is comprehensively reviewed by McDowall [1935]. However, in man, recent publications stress that in the upright position there is swelling of the legs with diminished blood flow through the lower half of the body [Grill, 1937; Ude, 1934; Youmans, Akeroyd & Frank, 1935]. At the same time there is a fall in blood volume due to the loss of plasma, which is accounted for by the swelling of the limbs [Thompson, Thompson & Dailey, 1928]. Ghrist [1933] found that abdominal support failed to improve cases of postural hypotension, and concluded that splanchnic vaso-dilatation was not the only factor producing the fall of blood pressure in the upright position. Ghrist [1930] and Roth [1937] studied the effects of splanchnic section in man, but did not find that this affected the circulatory response to posture.

These findings indicate that the reason for the imperfect compensation in man in the upright position is due to stagnation of blood in the lower limbs rather than in the splanchnic area. It therefore seemed expedient to re-examine the problem in animals.

### METHODS

Cats, under chloralose anaesthesia, were examined for the effect of the feet down position (F.D.) on the blood pressure [Edholm, 1940]. In series I, the effects were examined before and after removal of the

alimentary canal from the cardio-oesophageal junction to rectum, including pancreas and spleen (evisceration); in series II after the further removal of the liver.

In the evisceration the alimentary canal, together with the spleen and mesentery, was removed. The liver and kidneys were left in situ. The arterial supply to the gut was ligatured first and then the veins draining the gut, so reducing the volume of blood removed with the gut. The time taken for the whole operation was 2-5 min. In four experiments the gut was not removed after ligature of the blood supply, so as to test any possible effect that removal of the support given to the liver might have.

Removal of the liver was a longer procedure. In the cat the vena cava runs through the substance of the liver, which was removed by ligature of the different lobes which were then cut away leaving a small stump of liver tissue surrounding the vena cava.

In other animals, the effect of clipping off various vascular areas in the abdomen on the response to posture was studied, and in a further series the effect of haemorrhage was tested.

## RESULTS

The level of the systemic blood pressure is not markedly affected by evisceration, the average blood pressure before and after being  $135 \pm 8.1$  and  $128 \pm 10.0$  mm. Hg (14 exps.), there being an average fall of  $7.0 \pm 3.8$  mm. Hg.

Before evisceration, the average fall of blood pressure in the F.D. position is  $34 \pm 4.7$  mm. Hg initially, and  $30 \pm 6.9$  mm. Hg as the final fall before returning the animal to the horizontal. After evisceration the initial fall averaged  $35 \pm 5.3$  mm. Hg, but the final fall was increased to  $44 \pm 6.3$  mm. Hg. These results indicate a lack of compensation after evisceration, as the blood pressure continued to fall while in the F.D. position, instead of recovering slightly as occurred before evisceration (Fig. 1).

No significant difference was observed comparing the results obtained in the group of animals, the viscera of which were not removed after ligature of the blood vessels, with the others. The fall of blood pressure in the F.D. position after evisceration is not due to the removal of support from the liver.

Removal of the liver lowers the systemic pressure, the average before and after operation being  $131 \pm 11.9$  and  $106 \pm 10.4$  mm. Hg (7 exps.).

This operation was only carried out in animals which had been previously eviscerated. In the seven animals whose livers were successfully removed, the average fall of blood pressure in the F.D. position before hepatectomy was  $53 \pm 10.3$  mm. Hg initially and  $73 \pm 7.8$  mm. Hg

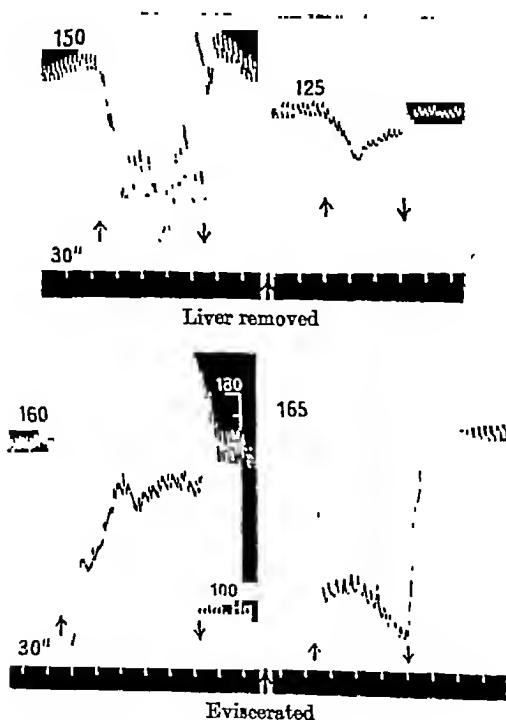


Fig. 1. The lower pair of tracings show the effect of the F.D. position on the blood pressure, before and after evisceration. Before evisceration, although there is a steep initial fall of pressure on placing the animal in the F.D. position there is a considerable degree of recovery before the animal is restored to the horizontal. After evisceration there is no such recovery. The upper pair of tracings show the effect of the subsequent removal of the liver. There is a very marked reduction in the fall of blood pressure in the F.D. position after the removal of the liver. The point of movement of the animal to and from the F.D. position is marked by arrows.

finally, but after operation the fall of pressure was only  $5 \pm 2.4$  and  $10 \pm 3.6$  mm. Hg respectively. The fall of pressure which was previously so marked was in every case almost completely abolished by the removal of the liver (Fig. 1). The abolition of the effect of gravity on the blood pressure is due to the absence of the liver and not to the accompanying fall of systemic blood pressure, as is shown by experiments in which a similar fall of blood pressure is produced by bleeding alone. Taking the blood

volume of the cat to be 1/15th body weight, eight animals were bled to an average of 12% of the total blood volume. This lowered the blood pressure from an average of  $130 \pm 6.4$  to  $119 \pm 5.9$  mm. Hg, but there was a marked increase in the fall of blood pressure in the F.D. position. Before haemorrhage, the initial and final falls were  $44.5 \pm 9.9$  and  $34.5 \pm 12.5$  mm. Hg respectively, after haemorrhage these increased to  $61.5 \pm 7.1$  and  $62.5 \pm 10.6$  mm. Hg. In five animals which were further bled to

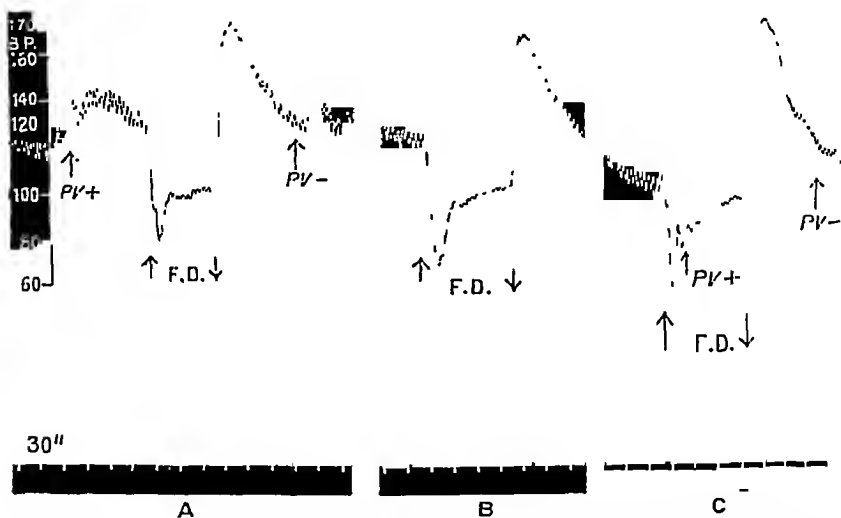


Fig. 2. The effect of clamping the portal vein is shown in these tracings. In A, the portal vein was clamped before tilting, and not released until the animal was restored to the horizontal. The initial fall of pressure on tilting and the recovery on replacing the animal in the horizontal are unaffected, as can be seen by comparing with B, which illustrates a control tilt with the portal vein intact. In C, the portal vein was clamped when the animal was in the F.D. position. Again the recovery of blood pressure on restoring the animal is unaffected. The arrows marked PV+ and PV- show the position of clamping and releasing the portal vein.

32% of their blood volume, the blood pressure fell to  $95 \pm 17.5$  mm. Hg, and the falls of pressure in the F.D. position were  $65 \pm 11.0$  mm. Hg initially and  $69 \pm 11.5$  mm. Hg finally.

These results indicate that the splanchnic area as a whole is not responsible for the fall of blood pressure which occurs in the F.D. position, but that the liver is primarily responsible. This conclusion is supported by the results of experiments in which various vascular areas were clipped off.

The inferior vena cava was clipped in two positions, above and below the entry of the hepatic veins, and the portal vein was also clipped. These procedures were carried out separately, and in one series the vessels were

clipped before tilting the animal, and in others when the animal was already in the F.D. position. The response of the animal to tilting was modified as follows.

Placing a clip on the vena cava below the entry of the hepatic veins with the animal in the horizontal, did not alter the fall of blood pressure on tilting. Clipping the portal vein similarly did not affect the result, (Fig. 2). On the other hand, clipping the vena cava above the junction of the hepatic veins and then tilting, almost abolished the fall of blood pressure in the F.D. position. The general level of blood pressure in all cases was lowered when the clips were put on, but most markedly by clipping the vena cava in the second position, i.e. above the entry of the hepatic veins. When the clips were put on with the animal in the F.D. position, there was no alteration of the recovery of blood pressure on replacing the animal in the horizontal with the inferior vena cava clipped below the hepatic veins. Clipping the portal vein did not markedly alter the blood pressure curve, but in some experiments the recovery of blood pressure on restoring the animal to the horizontal was not so abrupt as normal. Clipping the inferior vena cava above the hepatic veins prevented the blood pressure recovering on replacing in the horizontal. The blood pressure did not recover until the clip was removed (Fig. 3).

In other experiments the blood supply of the gut was clipped before tilting, and in other cases the clips were put on when the animal had been tilted. In all these experiments the gut was ligatured at the lower end of the rectum and round the gastro-oesophageal junction to diminish anastomotic connexions. This left the coeliac axis, the superior and inferior mesenteric arteries and the portal vein as the sole vascular connexions of the gut. When these vessels were clipped with the animal horizontal, there was a rise of blood pressure, which then gradually subsided to a steady level on an average slightly higher than before clipping. Tilting the animal resulted in the usual fall of pressure and recovery on replacing the animal in the horizontal (Fig. 4). In some experiments the fall of pressure on tilting was increased after clipping, but this increase did not exceed 10 mm. Hg. When the clips were put on with the animal in the F.D. position, there was a slight temporary rise of blood pressure; after this the blood pressure curve continued the previous tendency, i.e. either falling gradually or remaining stationary. The recovery on replacing the animal in the F.D. position was not significantly altered in most cases. In some experiments the recovery was not as rapid as before.

Finally, attempts have been made to remove the liver without removing the gut. This involves ligation of the portal vein and hepatic artery so converting the vascular area of the gut into a cul-de-sac, apart

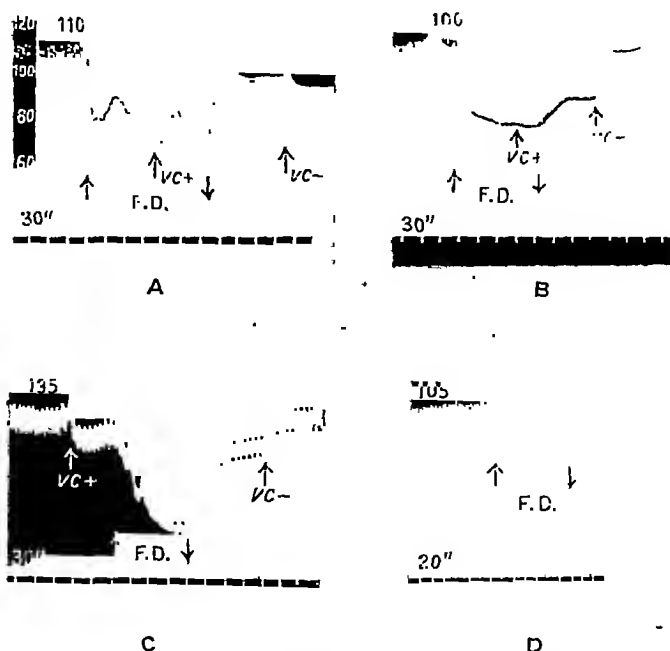


Fig. 3. A shows the effect of clamping the inferior vena cava below the liver, i.e. below the entry of the hepatic veins. The clamp was applied when the animal was in the r.p. position. In B the clamp was applied to the vena cava above the liver above the entry of the hepatic veins. In A the blood pressure recovers as usual on restoring the animal to the horizontal, in B there is only a slight rise of pressure on replacing the animal in the horizontal, and there is no recovery of pressure until the clamp on the vena cava is removed. In C the inferior vena cava was clamped below the liver before tilting, and not removed until the animal was replaced in the horizontal. The blood pressure curve is unaltered. In D the liver was removed, but the gut was left *in situ*. There is only a slight fall of pressure and no recovery on restoring to the horizontal. The arrows marked VC+ and VC- indicate the points at which the inferior vena cava was clamped and released.

from the small drainage via the haemorrhoidal veins and oesophageal veins. A fall of blood pressure follows ligation of the portal vein, of varying degree. Removal of the liver was carried out as described above. In several experiments the level of the blood pressure was so reduced to make it impossible to carry out any further experiment. However, some

successful experiments have been carried out and the results are substantially the same, as found when the liver is removed following evisceration, i.e. on tilting there is a slight gradual fall of blood pressure, on replacing the animal in the horizontal there is no recovery of the blood pressure.

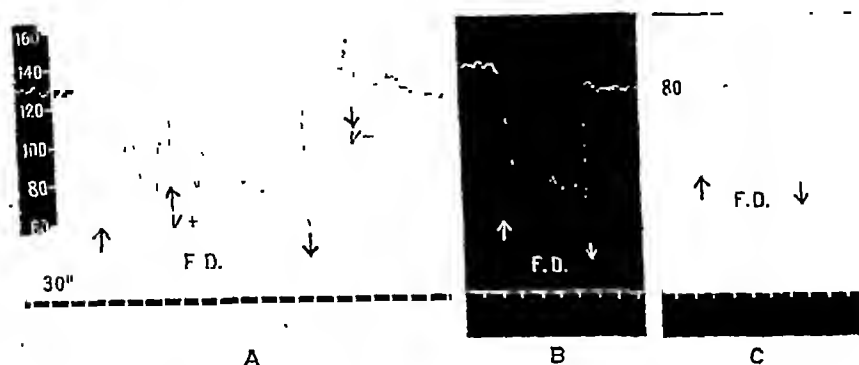


Fig. 4. A shows the effect of clamping off the blood supply of the gut. The clamps were put on when the animal was in the F.D. position, the blood pressure continues to remain low, but recovers as usual on restoring the animal to the horizontal, compared with the control tilt shown in B, where the circulation to the gut is intact. The arrows marked V+ and V- indicate the points at which the vessels were clamped and released. In C the inferior vena cava was clamped above the liver before tilting. This caused a marked fall of blood pressure (not shown in the tracing), but on tilting the animal into the F.D. position there is no further fall of pressure, and there is no rise on restoring to the horizontal.

## DISCUSSION

The fall of blood pressure which occurs after placing the animal in the F.D. position is sharp but of varying degree. There is usually a gradual recovery from this fall of pressure whilst the animal is kept in the F.D. position so that the final level of blood pressure is higher than the initial level just after tilting [Edholm, 1940].

Hill [1895] maintained that the fall of pressure was primarily due to blood accumulating in the splanchnic area and that the compensation which occurred was due to splanchnic vaso-constriction. But evisceration does not abolish the fall of pressure in the F.D. position, so the fall cannot be due to the accumulation of blood in the intestinal or mesenteric vessels. Moreover, clipping off the blood supply of the gut after the fall of blood pressure produced by tilting does not prevent the recovery of pressure on restoring to the horizontal. If the initial fall was due to the loss of blood in the splanchnic area, clipping the vessels would cut off



this extra volume of blood from the circulation and so should at any rate greatly diminish the recovery of blood pressure on restoration to the horizontal. After evisceration, compensation is diminished as the final level of pressure in the F.D. position is lower than the initial fall, i.e. the blood pressure continues to fall while the animal is kept feet down, instead of rising. Part of the compensatory mechanism at any rate is provided by splanchnic vaso-constriction.

When the liver is removed, the fall of blood pressure in the F.D. position is almost completely abolished, even in animals which previously had exhibited a very marked fall. This result was also obtained when the liver only was removed, leaving the gut intact. In no case was there a sharp initial fall of pressure, any fall which does occur develops gradually while the animal is kept in the F.D. position. These results are not due to the lower level of systemic blood pressure produced by removal of the liver, as it has been previously shown that at lower levels of blood pressure animals exhibit greater falls in the F.D. position [Edholm, 1940]. This is confirmed by the increased effect of tilting after haemorrhage. The effects of clipping the portal vein and the vena cava above and below the entry of the hepatic veins, show that it is only when the hepatic blood is cut off from the heart that the blood pressure response to tilting is reduced.

It is concluded that the fall of blood pressure in the cat in the F.D. position is due to the accumulation of blood in the liver, not in the splanchnic area as a whole. The slow gradual fall of pressure which occurs in the F.D. position in hepatectomized cats is probably due to the accumulation of blood in the lower limbs, as has been demonstrated in man. This factor is of greater importance in man than in cats, owing to the different relative proportions of the lower limbs.

The changes of blood pressure produced by tilting appear to be due to alteration of the venous return of blood to the heart. The fall of blood pressure can be prevented by clipping the inferior vena cava above the entry of the hepatic veins, although clips on the same vessel lower down or on the portal vein do not have this effect. Similarly the recovery of blood pressure on restoring the animal to the horizontal can be prevented. Hill [1895] observed the increased blood flow in the thoracic inferior vena cava on replacing the animal after tilting. This blood comes in large part from the liver. Sjöstrand [1934] has shown that the liver can hold a large volume of blood, up to 20 % of the total blood volume in the dog.

The fall of blood pressure in the F.D. position in animals with the splanchnic circulation intact can be overcome by abdominal compression,

as first shown by Hill [1895] and confirmed by many others. These effects Hill considered to be due to the splanchnic area, but they can also be obtained in the eviscerated animal. The effect is more easily explained by pressure on the liver. The increase in depth of respiration which has been observed when the animal is kept in the F.D. position [Mark & Neumann, 1932] and also seen in the course of the present investigation, is particularly noticeable when the fall of blood pressure is marked. The respiration is then gasping in character, and associated with strong contractions of the abdominal muscles, which produce a rise of blood pressure. As soon as the abdominal muscles relax again the blood pressure falls. The simultaneous contraction of the diaphragm and abdominal muscles squeezes the liver and forces blood up the vena cava, increasing the venous return.

The effect of haemorrhage is to increase the fall of blood pressure in the F.D. position and to diminish the powers of compensation. After haemorrhage there is splanchnic vaso-constriction, including contraction of the spleen, so no further constriction can take place to compensate for the fall of blood pressure in the F.D. position. These findings support the view that the fall of blood pressure in the F.D. position is not due to the collection of blood in the splanchnic area, as this area is in a state of vaso-constriction owing to the haemorrhage, and yet the fall of blood pressure in the F.D. position is markedly increased.

It is concluded that the fall of blood pressure in the F.D. position in the cat is due to the accumulation of blood in the liver which is normally compensated by splanchnic vaso-constriction.

#### SUMMARY

1. The mechanisms in the cat responsible for the fall of blood pressure in the feet-down (F.D.) position, have been examined.
2. Evisceration does not abolish the fall, but diminishes the vascular compensation.
3. Removal of the liver, both after evisceration and independently, almost abolishes the fall of blood pressure in the F.D. position.
4. The reason for the fall of blood pressure is not the collection of blood in the splanchnic area, but in the liver. The splanchnic area is partly responsible for the compensation following this fall.
5. The recovery of blood pressure on restoring the animal to the horizontal is due to the return to the right side of the heart of the accumulated blood in the liver.

I wish to thank Prof. R. J. S. McDowall and Prof. H. Barcroft for their help and advice in this investigation. A large part of the experimental work was carried out in the Physiology Department, King's College, London, during the tenure of a Bovril Research Scholarship.

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## THE EFFECT OF PARTIAL PANCREATECTOMY ON THE CONCENTRATION OF INSULIN IN THE PANCREATIC REMNANT<sup>1</sup>

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EXTENSIVE partial pancreatectomy leads to degranulation of the beta cells of the islets of Langerhans [Homans, 1913, 1914] followed by hydropic degeneration of these cells [Allen, 1913, 1922]. Allen showed that the severity of the diabetes in the animals was related to the extent of the beta cell changes. He believed that the degenerative changes in the beta cells were the result of excessive islet activity, i.e. that the beta cells became exhausted through overwork. The results of our insulin assays support this interpretation.

### METHODS

Dogs of both sexes were used. Pancreatic tissue was removed under nembutal anaesthesia, and the insulin content determined by the method previously described [Best, Haist & Ridout, 1939].

### EXPERIMENTS

*The distribution of insulin in normal dog pancreas.* Before investigating the effects of partial pancreatectomy in dogs, an attempt was made to find the distribution of insulin in the pancreas of normal animals. Five dogs were anaesthetized with nembutal and the pancreas of each was removed and divided into three portions. These parts were weighed and assayed separately. They were: (1) the free splenic end of the pancreas (tail), (2) the attached duodenal portion (body), and (3) the free duodenal end (head). The insulin concentrations, expressed as units of insulin per g. pancreas, together with other data for these animals, are

<sup>1</sup> A preliminary note on this work appeared in *Science*, 91, 410 (1940).

TABLE 1.

Dog	Sex	Weight kg.	Total pancreas wt. g.	Total in- sulin in pancreas units	Units of insulin/g. pancreas				Units per kg. dog wt.
					Head	Body	Tail	Weighted average for whole pancreas	
A	♀	7.0	14.5	32.7	1.3	2.1	3.2	2.3	4.7
B	♀	5.5	16.6	54.8	2.7	3.0	4.1	3.3	10.0
C	♂	8.2	15.4	46.6	2.4	3.0	3.8	3.0	5.7
D	♂	10.5	20.1	73.4	2.6	3.5	4.7	3.7	7.0
E	♀	10.7	20.4	76.0	1.9	4.1	5.1	3.8	7.2
Average					2.2	3.1	4.2	3.2	

given in Table 1. The average results are better illustrated in Fig. 1. This figure shows a definite difference in the insulin concentration in different portions of the organ and indicates that an insulin assay made on biopsy specimens taken from the pancreas would not give accurate information on the insulin concentration of the remaining tissue.

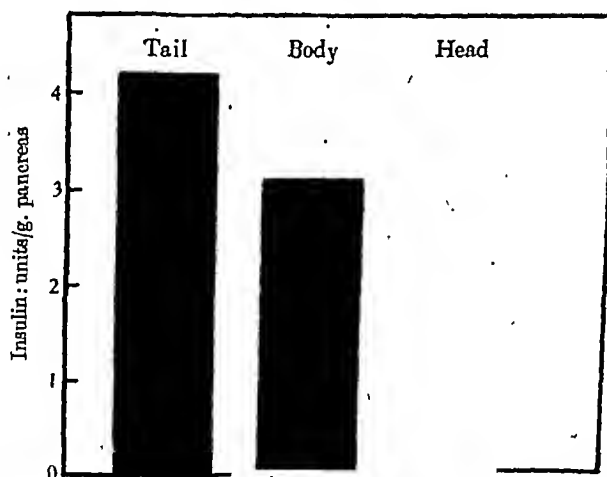


Fig. 1. Distribution of insulin in dog pancreas. Tail, free splenic end. Body, attached duodenal portion. Head, free duodenal end.

*Partial pancreatectomy.* In the experiment on partial pancreatectomy different amounts of pancreas were removed. The portion left was in the region of the main duct where it enters the duodenum. When the remnant was not too small the animal did not become diabetic and the remaining pancreatic tissue had a concentration of insulin which was usually within the expected normal range (Table 2).

TABLE 2

Dog	Init. wt. of dog kg.	Wt. of pancreas		Interval between operations days	Units of insulin per g. pancreas	
		First portion g.	Remnant g.		First portion	Remnant
D <sub>A</sub>	20	14.0	8.9	31	4.1	3.6
D <sub>B</sub>	19	14.3	9.4	31	2.2	1.8
D <sub>C</sub>	20	12.8	13.9	30	2.4	1.5
D <sub>4</sub>	9	—	1.3	34	—	2.4
D <sub>5</sub>	10	18.9	9.0	11	2.8	3.8
D <sub>12</sub>	18	21.9	19.0	7	4.5	2.8
D <sub>13</sub>	10	21.0	2.0	20	2.6	2.1
D <sub>14</sub>	12	34.8	8.3	26	—	2.1
D <sub>16</sub>	18	20.7	16.8	3	3.5	2.8
D <sub>18</sub>	15	23.5	17.4	3	2.6	2.4

Two animals deserve special mention. Dog D<sub>2</sub> had only a very small remnant left (1.3 g.). This animal showed a transient diabetes but recovered in 6 days. Thirty-four days after the partial pancreatectomy was performed the pancreas had a normal concentration of insulin. Dog D<sub>15</sub> became diabetic but then stopped eating. The hyperglycaemia gradually became less and on the ninth day after operation the fasting blood sugar was again normal. The insulin concentration in the pancreas of this animal was normal. The findings in these animals illustrate the fact that even though the pancreatic remnant is very small, if the animal does not become diabetic, or if it recovers from the diabetes, the insulin concentration in the remnant may be within normal limits.

When sufficient pancreas was removed, the animals became diabetic, as judged by the high level of the fasting blood sugar and the glycosuria. In these animals the insulin concentration in the pancreatic remnants was very low (Table 3). A comparison of the averages of the insulin

TABLE 3

Dog	Initial wt. of dog kg.	Wt. of pancreas		Interval between operations days	Units of insulin per g. pancreas	
		First portion g.	Remnant g.		First portion	Remnant
D <sub>4</sub>	9	26.8	2.3	10	2.1	<0.11
D <sub>5</sub>	10	23.9	3.4	11	2.4	<0.15
D <sub>9</sub>	11	—	4.8	9	—	0.14
D <sub>1</sub>	13	—	3.3	7	—	<0.12
D <sub>10</sub>	13	29.5	2.6	9	3.6	0.39
D <sub>11</sub>	12	24.3	3.7	9	4.5	0.02
D <sub>13</sub>	10	21.0	0.4	9	—	<0.18

concentration in the pancreatic remnants of diabetic and non-diabetic animals shows that when sufficient pancreas is removed to make the animals diabetic, the insulin concentration in the remnants falls to

very low values (average, less than 0.16 unit/g.), whereas when enough is left so that the animal does not become diabetic, the insulin concentration in the remnant stays within the normal range. The average value for the insulin concentration in the pancreatic remnants of the animals which did not become diabetic was 2.6 units/g.

*Partial pancreatectomy in hypophysectomized dogs.* Partial pancreatectomy has been performed successfully in two hypophysectomized dogs. The results are given in Table 4. In one of the hypophysectomized

TABLE 4

Dog	No. 86	No. D <sub>5</sub>
Initial wt. (kg.)	7	12
Days after hypophysectomy when partial pancreatectomy performed	19	34
Time after partial pancreatectomy when remnant taken (days)	32	8
Wt. of pancreas removed:		
First portion	12.1	14.9
Remnant	6.3	0.7
Units of insulin per g. pancreas:		
First portion	2.3	4.9
Remnant	2.0	3.5

animals (no. 86) a relatively large portion of the pancreas was left. As would be expected, the animal did not become diabetic and the insulin concentration in the remnant showed very little change. The whole pancreatic remnant was eventually removed from this animal but no insulin was required until 74 days later. In the other dog (D<sub>5</sub>) only a very small remnant of pancreas was left. Again, as might be expected, the animal did not become diabetic and the insulin concentration in the remnant (0.7 g.) remained high. In this dog the average fasting blood sugar prior to hypophysectomy was 80 mg. %; after hypophysectomy but before the partial pancreatectomy it was 60 mg. %. The portion of pancreas was removed 34 days after the hypophysectomy, and on the eighth day following the operation the fasting blood sugar was only 48 mg. %.

The result in this latter animal shows that the insulin concentration in the pancreas of an hypophysectomized, partially depancreatized dog may be maintained at a normal level even though only a very small remnant of pancreas is left.

#### DISCUSSION

When part of the pancreas is removed from a dog and the remnant is not too small, the insulin content remains within the expected normal limits. In some cases diabetes does not appear when only a small amount

of pancreatic tissue is present. Since the animal is not diabetic it is probable, under these particular conditions, that the total liberation of insulin has not been greatly reduced, and since the insulin content of pancreas has not fallen we may believe that production is able to keep up with liberation. Since a small fraction of the pancreas is doing the work which the whole gland had previously performed it seems logical to assume that the rate of production and liberation of insulin in the remaining pancreatic tissue has increased. This apparently can occur without any very great change in insulin concentration.

When the size of the remnant is still further reduced, a point is reached beyond which production can no longer keep up with liberation and the insulin concentration in the pancreatic remnant falls. As a result of the excessive activity a degranulation of the beta cells occurs and finally hydropic degenerative changes in the islet cells appear [Allen, 1913, 1922; Homan's, 1913, 1914].

The pancreatic changes following extensive partial pancreatectomy are similar to those resulting from the administration of anterior pituitary extracts. Consequently it is important to determine whether or not the change in the insulin concentration of the remaining pancreas, in partially depancreatized animals, is due to the action of a pituitary substance. This question cannot be answered at present. In one experiment in which an extremely small pancreatic remnant was left in an hypophysectomized dog, no great reduction in insulin concentration was observed. This does not prove that a pituitary substance is directly responsible for the changes observed when the pituitary is intact. It does, however, support other evidence which indicates that in the absence of the pituitary, heavy demands on the pancreas for insulin are not made.

#### SUMMARY

1. The concentration of insulin was found to be greatest in the free splenic end of the dog's pancreas (4.2 units/g.), next in order in the attached duodenal portion (3.1 units/g.) and least in the free duodenal end (2.2 units/g.).
2. In partially depancreatized animals, when a sufficient amount of the gland was removed to produce diabetes, the insulin concentration in the remnant was reduced to very low values (average, less than 0.16 unit/g.), whereas when enough gland was left to prevent the onset of diabetes, the insulin concentration in the remnant was usually found to be within the expected normal range (average 2.6 units/g.).



3. In two hypophysectomized, partially depancreatized dogs, one of which had only a very small remnant of pancreas, the insulin concentration was within normal limits.

4. The results of these experiments support Allen's conclusion that the changes in the islet cells of the pancreatic remnant after extensive partial pancreatectomy are due to overstrain.

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## THE EFFECT OF INSULIN AND ANTERIOR PITUITARY EXTRACT ON THE INSULIN CONTENT OF THE PANCREAS AND THE HISTOLOGY OF THE ISLETS<sup>1</sup>

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It has been shown previously that the administration of extracts of anterior pituitary gland caused (1) a profound reduction in the insulin content of the pancreas [Best, Campbell & Haist, 1939], (2) degranulation, and hydropic degeneration of the beta cells of the islets of Langerhans [Richardson & Young, 1938; Ham & Haist, 1939, 1941], and (3) proliferative responses in many tissues, including the ducts, acini and islets of the pancreas [Ham & Haist]. It was important to find whether or not the administration of insulin along with the extract would prevent these changes. The results of such an experiment throw some light on the mechanism by which anterior pituitary extract (A.P.E.) causes islet damage and loss of pancreatic insulin.

### METHODS

The methods used for the estimation of the insulin content of the pancreas, sugar in blood and urine [Best *et al.* 1939], and the preparation of the A.P.E. [Campbell & Keenan, 1940] have been described. For the histological studies small blocks of pancreatic tissue were removed at operation, fixed in Zenker-formol solution and sectioned in paraffin at 3 $\mu$ . The sections were stained for islet granules by the method of Bowie [1924] and in addition by the Mallory-azan and haematoxylin-eosin technique.

<sup>1</sup> A preliminary note on this subject appeared in *Proc. Amer. Physiol. Soc.* 129 (1940).  
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The animals were fed a total of 400 g. lean meat and 40 g. dextrose per day, given in two meals at 9 a.m. and 4 p.m. Three experiments were undertaken, in all of which the dogs were fed and given daily subcutaneous injections of A.P.E. In each experiment two groups of animals were used. The members of one group received injections of regular insulin and protamine zinc insulin<sup>1</sup> each day. The injection periods lasted for 11, 7, and 7 days.

### RESULTS

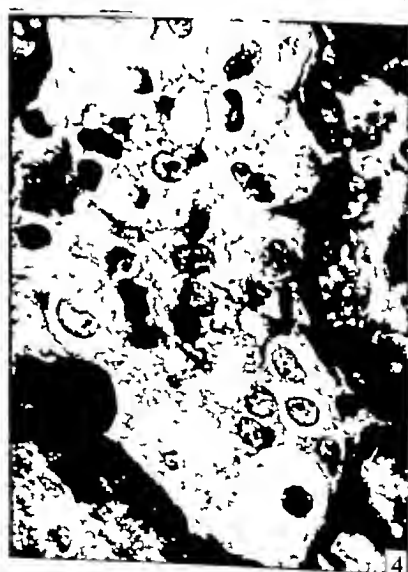
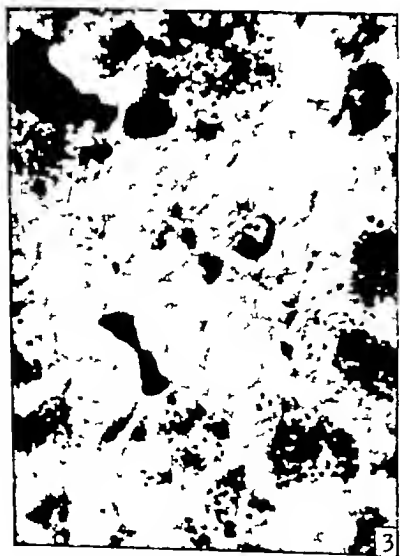
*Exp. 1.* Six dogs were given A.P.E. for 11 days. Three of these also received daily injections of protamine zinc insulin. Of the three animals which received no insulin, two (nos. 221 and 125) showed glycosuria and hyperglycaemia (Table 2A). Using the same criteria, the other dog (no. 220) was almost completely resistant to the A.P.E. In the group given insulin the glycosuria was well controlled in the last 4 days, although in dogs no. 217 and 218, definite sugar loss occurred. In Table 1A the insulin content of the pancreas in the treated and control animals is shown. In two of the dogs given A.P.E. alone, the insulin concentration of the pancreas was very low. No. 220, which was resistant to the extract, had an insulin content within the normal range.

In those animals which received insulin in addition to the extract the insulin content of the pancreas, though somewhat less than normal, was much higher than would be expected if the A.P.E. had been given alone.

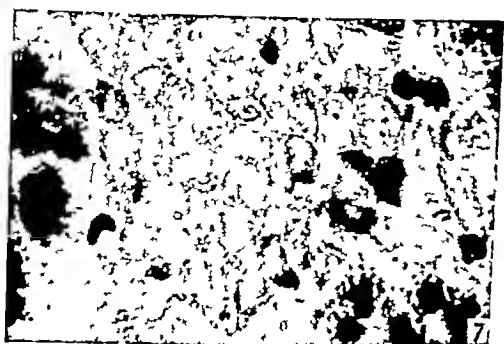
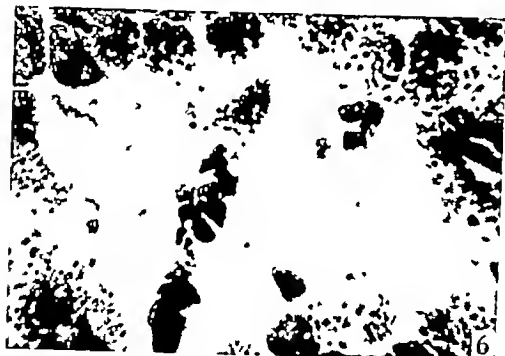
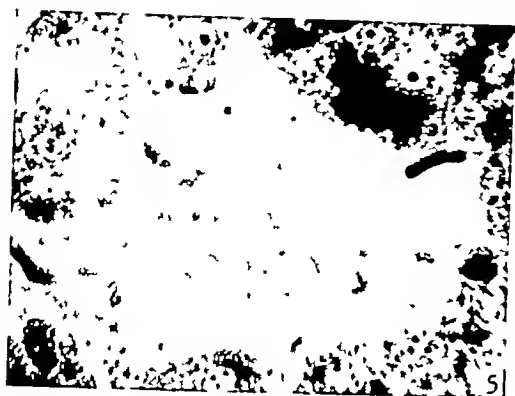
Sections of the pancreas revealed the following facts. In the group given A.P.E. alone, dog 220, which was not responsive to the extract, showed almost normal beta cell granulation (Pl. 1, fig. 1). In the two other dogs of this group, which received the extract but no insulin, degranulation and hydropic degeneration of the beta cells was evident. The changes in the beta cells were accompanied by hydropic changes in the small ducts of the pancreases of these animals. An islet from one of the susceptible dogs (no. 221) is illustrated in Pl. 1, fig. 2.

Of the three dogs which received insulin and A.P.E. for 11 days, two showed fairly good beta cell granulation (one of these is illustrated in Pl. 1, fig. 3) but the granulation was defective in dog 218. The pancreas of this dog had a lower content of insulin per g. tissue than either of the others in the insulin-injected group. Mitotic figures in both acinar and islet cells were found in the dogs that received the extract alone and also in those that received the extract and insulin. The proliferative effect on the pancreas of an animal receiving insulin in addition to the extract is

<sup>1</sup> From the Connaught Laboratories.



Figs. 1-4.



Figs. 5-9.

TABLE I

No.	Group	No. of days injected	Initial wt., dog	Wt. pancreas	Initial/total pancreas	Uddg./g. pancreas	Unit/gg. body wt.	Average daily dose, i.u./g.	Average daily dose, % cal.	Average daily dose regular insulin
A 217	A.P.R. + Insulin	11	0.2	17.54	40.0	2.3	0.5	90	17.0	—
218	" "	11	12.8	30.05	61.1	1.5	1.8	117	51.4	—
219	" "	11	17.4	51.00	14.0	2.2	0.0	180	47.0	—
220	A.P.R. only	11	14.3	40.70	102.0	4.1	13.4	101	—	—
221	" "	11	12.0	38.25	14.0	0.38	1.2	125	—	—
225	" "	11	8.2	22.2	7.3	0.33	0.87	170	—	—
B 238	A.P.R. + Insulin	7	7.0	17.55	45.8	2.0	0.6	119	30.4	—
239	" "	7	12.1	27.03	70.4	2.0	5.7	178	33.1	—
240	" "	7	11.8	33.00	74.8	2.3	0.3	158	30.7	—
241	" "	7	12.0	40.11	9.30	0.23	0.73	105	50.5	—
242	A.P.R. only	7	10.8	29.24	12.2	0.17	1.1	157	—	—
243	" "	7	0.3	28.05	10.7	0.37	1.2	130	—	—
244	" "	7	11.7	28.00	4.01	0.14	0.35	155	—	—
245	" "	7	10.0	25.10	8.76	0.35	0.88	128	—	—
251	A.P.R. only	7	10.0	27.5	5.8	0.21	0.53	185	—	—
252	" "	7	10.2	27.0	0.4	0.23	0.03	102	—	—
253	" "	7	9.7	23.7	3.0	0.18	0.40	230	—	—
C 270	A.P.R. + Insulin	7	0.0	30.28	10.35	0.34	1.1	95	50.0	0.3
277	" "	7	12.0	31.17	38.7	1.13	3.1	125	20.0	—
278	" "	7	7.3	14.00	0.0	0.05	1.3	70	45.5	3.4
279	" "	7	10.0	25.01	07.5	2.0	0.1	90	20.2	2.3
280	A.P.R. only	7	0.0	10.84	1.00	0.00	0.2	33	—	—
281	" "	7	11.4	27.07	2.8	0.10	0.2	100	—	—
282	" "	7	10.0	30.40	8.8	0.21	0.81	97	—	—

Range in six normal animals, 2.3-3.0 unit/gg. pancreas, or 1.7-30.0 unit/gg. body weight.

TABLE 2

Dog No.	Treatment*	Daily blood sugar 16 hr. after last meal											Sugar excretion average g.		
		0	1	2	3	4	5	6	7	8	9	10		11	
A	A.P.E. + P.Z.I.	103	94	121	—	182	265	256	201	104	176	102	106	4th to 7th day, 12.3	
	"	95	112	123	—	90	113	170	190	166	326	107	87	2nd to 9th day, 7.8	
	"	87	117	95	—	87	106	114	106	107	102	83	77	1st to 11th day, 1.0	
	A.P.E.	132	88	116	—	102	106	114	—	117	104	106	100	2nd to 11th day, 0.5	
	"	107	140	196	—	110	120	286	246	286	340	201	375	2nd to 11th day, 57.2	
B	"	90	117	193	—	273	—	102	—	219	—	112	114	8th to 11th day, 16.2	
	A.P.E. + P.Z.I.	55	117	107	120	137	103	120	123	—	—	—	—	2nd to 7th day, 0.6	
	"	51	58	73	114	121	112	109	84	—	—	—	—	2nd to 7th day, 1.6	
	"	45	100	119	129	120	112	120	106	—	—	—	—	2nd to 7th day, 1.6	
	"	88	103	113	135	145	135	133	123	—	—	—	—	2nd to 7th day, 11.4	
	A.P.E.	92	103	116	121	126	121	121	117	—	—	—	—	2nd to 7th day, 4.6	
	"	109	114	133	162	174	132	110	114	—	—	—	—	2nd to 7th day, 11.6	
	"	106	123	144	200	240	222	218	204	—	—	—	—	2nd to 7th day, 48.5	
	"	109	143	252	244	303	308	348	339	—	—	—	—	2nd to 7th day, 65.1	
	121	"	104	119	132	130	—	196	195	182	—	—	—	2nd to 7th day, 3.5	
	124	"	109	130	144	139	—	266	266	198	—	—	—	5th to 7th day, 56.0	
	173	"	97	130	—	210	283	—	270	195	—	—	—	2nd to 7th day, 11.2	
	C	A.P.E. + P.Z.I. + R.I.	91	100	104	156	228	140	88	109	—	—	—	—	2nd to 7th day, 9.4
		"	87	67	88	88	117	94	84	109	—	—	—	—	2nd to 7th day, 0.0
"		97	102	126	112	123	177	135	130	—	—	—	—	2nd to 7th day, 3.9	
"		92	77	94	106	112	88	91	124	—	—	—	—	2nd to 7th day, 0.9	
A.P.E. only		91	121	253	285	294	296	405	487	—	—	—	—	2nd to 7th day, 4.7	
"		95	85	94	208	260	183	209	279	—	—	—	—	2nd to 7th day, 23.6	
"		88	88	102	112	137	124	135	186	—	—	—	—	2nd to 7th day, 6.5	

\* P.Z.I. = protamine zinc insulin.

R.I. = regular insulin.

Sugar excretion average g.

4th to 7th day, 12.3  
 2nd to 9th day, 7.8  
 1st to 11th day, 1.0  
 2nd to 11th day, 0.6  
 2nd to 11th day, 57.2  
 8th to 11th day, 15.2  
 2nd to 7th day, 0.6  
 2nd to 7th day, 1.6  
 2nd to 7th day, 1.6  
 2nd to 7th day, 11.4  
 2nd to 7th day, 4.6  
 2nd to 7th day, 11.6  
 2nd to 7th day, 48.5  
 2nd to 7th day, 65.1  
 2nd to 7th day, 3.5  
 5th to 7th day, 50.0  
 2nd to 7th day, 11.2  
 2nd to 7th day, 9.4  
 2nd to 7th day, 0.0  
 2nd to 7th day, 3.9  
 2nd to 7th day, 0.9  
 2nd to 7th day, 4.7  
 2nd to 7th day, 23.6  
 2nd to 7th day, 6.5

illustrated in Pl. 2, figs. 8 and 9. Marked proliferative changes in the thyroid gland and the adrenal cortex were observed in all the animals used in this experiment. The results of the insulin assays and the examination of the histological changes in the 11-day animals seem to indicate that the administration of insulin with the pituitary extract tends to hinder the fall in the insulin content of the pancreas and tends also to prevent the degranulation and marked hydropic change in the islet cells.

Previous investigations had shown that, in dogs, hydropic degeneration of the beta cells of the islets was very severe after 11 days of treatment with A.P.E. After 7 days the hydropic degeneration, while evident, was usually not extensive. For this reason an 11-day injection period was desirable. However, at 7 days the insulin content of the pancreas was very low and it became increasingly difficult to maintain the animals during longer periods of injection. Young [1938] found that, using a constant dose, the maximum effect of the injection of A.P.E. on blood sugar and glycosuria was reached about the seventh day. The effect then began to decrease. There might also be some objection to the results of the first experiment on the ground that the animals in the insulin-treated group became unresponsive to the extract by means of mechanism unrelated to the insulin administration. It was therefore decided to use a 7-day injection period in the following experiments.

*Exp. 2.* In this experiment four dogs were given seven daily injections of the A.P.E., and four were given seven daily injections of both A.P.E. and protamine zinc insulin. Results obtained in three other dogs treated with A.P.E. alone are also included. All seven animals receiving A.P.E. alone showed hyperglycaemia and glycosuria (Table 2B) in varying degrees. The insulin content of the pancreas in all these dogs was less than 0.5 unit/g. pancreas, averaging 0.28 unit/g. (Table 1B).

In the group given insulin in addition to the A.P.E. one animal exhibited an uncontrolled diabetes, although up to 70 units of protamine zinc insulin were given per day. The insulin content of the pancreas in this animal (no. 241) was very low (0.23 unit/g. pancreas). In the other animals of this group the hyperglycaemia and sugar excretion were fairly well controlled by the insulin (Table 2B). In these animals the insulin content of the pancreas (2.6, 2.6 and 2.2 units/g. pancreas in dogs 238, 239 and 240 respectively) is much higher than in the group given A.P.E. alone. In a group of six normal dogs the insulin content of the pancreas ranged from 2.3 to 3.9 units/g. pancreas.

Histological studies of the pancreases of these animals revealed that extensive degranulation of the beta cells occurred in all four dogs



injected with the extract alone. Beta-cell granulation was moderately good in two of the animals which received both extract and insulin (dogs 238 and 239) but there was some depletion of beta-cell granules in dog 240. In dog 241 insulin failed to control the diabetes, and the islets exhibited beta-cell degranulation (see Pl. 2, fig. 6). Proliferative effects of the extract on the various epithelial elements in the pancreas and on the thyroid gland and adrenal cortex were observed in all these animals.

As in the first experiment, the results of both the insulin assays and the histological studies indicate that insulin decreases the ability of anterior pituitary extracts to reduce the insulin content of the pancreas and cause beta-cell degranulation. In this series the hyperglycaemic and glycosuric effects of the extract were well prevented in certain of the animals which received insulin but it might be argued that these dogs were unresponsive to the extract and that this would account for our results. Inspection of the values of blood and urinary sugars indicated that all the dogs of the last series were responsive to the A.P.E. but we decided to conduct a similar experiment, using dogs which had been previously tested for their response to the extract.

*Exp. 3.* A number of dogs were tested for their response (hyperglycaemia and glycosuria) to A.P.E. Seven sensitive animals were selected and divided into two groups (of four and three dogs) by two independent observers, so that the average sensitivity of the groups to A.P.E. was approximately the same. Seven daily injections of A.P.E. were given to all the animals but those of one group received, in addition, injections of protamine zinc and ordinary insulin.

The curves showing the sugar excretion in these two groups are given in Text-fig. 1. Dog 280, given A.P.E. alone, was extremely diabetic, ceased to eat on the fifth day and consequently received a reduced dosage and only six injections of extract. This might account for the diminished sugar excretion, although the fasting blood sugar level was still very high (Table 2C). The two other dogs in this group were also diabetic.

Of the animals given insulin and A.P.E., dog 276 developed glycosuria on the last 4 days, and dog 278 on the third, sixth and seventh days; dogs 277 and 279 were fairly well controlled by the insulin.

The insulin content of the pancreas was very low in all the animals which received A.P.E. alone. The animals receiving insulin in addition to A.P.E. did not show such a great reduction (Table 1C). The data indicate that the insulin content of the pancreas was highest in the animals which were best controlled by insulin. Histological studies of the pancreases of the three dogs injected with the extract alone revealed.

that extensive degranulation of the beta cells had occurred, and in two of the dogs (nos. 280 and 281) this was associated with severe hydropic degeneration of the islets. Hydropic degeneration in dog 280 is illustrated in Pl. 1, fig. 4, and degranulation of beta cells in Pl. 2, fig. 5. Of the dogs which received insulin in addition to the extract, the islets of one (no. 276) whose diabetes was not controlled by the insulin, showed severe degranulation of the beta cells, and presented a picture similar

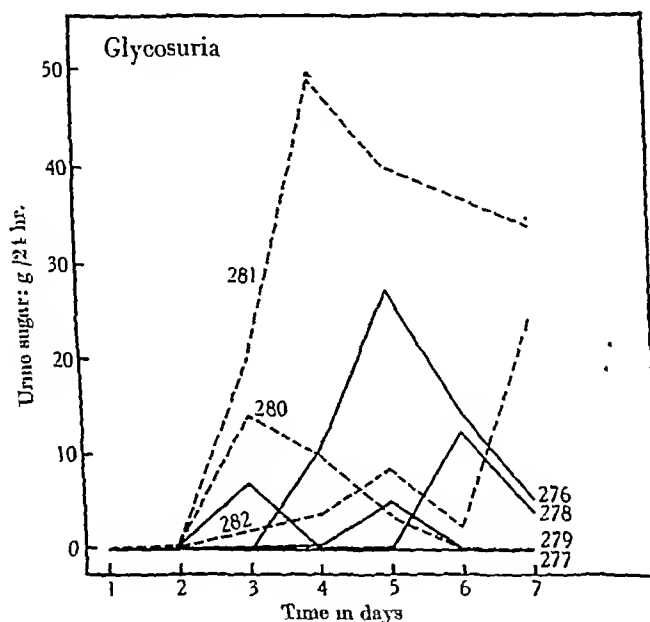


Fig 1. Insulin and anterior pituitary extract --- A.P.E. alone.  
 — A.P.E. plus insulin.

to that of dog 241 (see Pl. 2, fig. 6). The insulin content of the pancreas in this animal was lower than normal. Of the remaining three dogs in this group, dogs 277 and 279 exhibited beta granulation to a moderate degree. As in the preceding experiments, the proliferative effects of the extract in the various epithelial elements of the pancreas and in other endocrine glands were observed in all the animals.

### DISCUSSION

The results of the present series of experiments confirm the previous findings that the administration of A.P.E. to normal, fed dogs produces a great reduction in the insulin content of the pancreas. The effect was usually accompanied by a considerable hyperglycaemia and glycosuria.

The reduction of insulin content was associated with a degranulation and finally a hydropic degeneration of the beta cells of the pancreatic islets.

These results provide confirmation for the theory that A.P.E. damages beta cells and reduces their insulin content by stimulating them to overwork. The suggestion that beta cells can be stimulated to such a degree that they degenerate and die was first made by Allen [1922]. He induced overwork of beta cells by removing most of the pancreas and leaving only a small fragment to supply the needs of the animal. After insulin became available, Copp & Barclay [1923] and Bowie [1926] confirmed the overwork hypothesis by showing that the degenerative changes in a pancreatic fragment could be prevented or alleviated by the administration of insulin. The first evidence that A.P.E. injures beta cells by causing them to overwork was obtained by Richardson and Young. They found examples of degranulation and hydropic degeneration of beta cells in two animals which were receiving daily injections of A.P.E. Best *et al.* [1939] found that the insulin content of the pancreas became progressively reduced when daily injections of A.P.E. were given. Ham and Haist noted, in the same animals, a progressive degranulation of beta cells followed by hydropic degeneration, i.e. the same sequence of histological events observed by Allen in pancreatic fragments. In the present study it has been found that the administration of insulin with A.P.E. tends to prevent the hyperglycaemia and glycosuria, the reduction in insulin content, and the degenerative islet changes. In certain animals which were not responsive to the extract the insulin content of the pancreas did not fall, and in those not adequately controlled by the large amounts of insulin given, the fall in the insulin content of the pancreas was not prevented. When all the results are included, the average value for the insulin content of the pancreas in the group receiving A.P.E. alone was 0.55 unit of insulin per g. pancreas, while that for the group receiving insulin in addition to the A.P.E. was 1.7 units of insulin per g. pancreas. If the results in those animals which did not respond well to the extract or were not controlled by insulin are omitted, then the average value for the group receiving A.P.E. was 0.26 unit of insulin per g. pancreas, and that for the group receiving insulin in addition to A.P.E. was 2.1 units per g. pancreas. Both methods of presentation indicate that insulin administration tends to prevent the reduction in the insulin content of the pancreas resulting from the administration of A.P.E. The histological studies show that the administration of insulin tends to inhibit degranulation of the beta cells and prevent the hydropic degeneration of these cells. The finding that the administration of

insulin largely prevents the fall in insulin content and the degranulation and hydropic degeneration of the beta cells of the islets supports the view that the chief way in which A.P.E. injures beta cells is by causing them to overwork.

The mechanism by which insulin prevents overwork of the beta cells is a matter for speculation. If insulin secretion is regulated by the blood-sugar level, insulin may lessen the strain on beta cells by lowering the blood sugar. If, however, insulin secretion is regulated by the amount of insulin in the blood, it may be that injected insulin lessens the strain on the beta cells by raising the level of blood insulin.

In these experiments it was noted that the stimulus for the proliferation of the cells of the pancreas provided by the administration of A.P.E. was not eliminated by the injection of insulin. If the proliferative changes can be obtained in diabetics of long standing, then there is the possibility that the simultaneous administration of pituitary extract together with adequate amounts of insulin may increase the number of beta cells and thus improve islet function.

Another important observation was that there is very close correlation between the insulin concentration of the pancreas and the degree of beta cell granulation, as shown by Bowie's stain. When the beta cells were well filled with granules the insulin concentration was high, and when the beta cell granules were reduced in number the insulin concentration was low. This finding again emphasizes the importance of the beta cells in insulin production.

#### SUMMARY

Dogs receiving daily injections of anterior pituitary extract (A.P.E.) for 7 or 11 days show a reduction in the insulin content of the pancreas, accompanied by degranulation and hydropic degeneration of the beta cells of the islets of Langerhans. The daily administration of protamine zinc insulin along with the A.P.E. tends to prevent the reduction in insulin content of the pancreas and the degranulation and hydropic degeneration in the beta cells of the islets of Langerhans. These findings support the conclusion that the reduction in insulin content of the pancreas and the islet cell changes resulting from A.P.E. administration are brought about by overwork.

The proliferative changes in the pancreas and other tissues resulting from the injection of A.P.E. were not prevented by administration of insulin in these experiments.

The insulin content of the pancreas was closely correlated with the degree of granulation evident in the beta cells of the islets of Langerhans.

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## EXPLANATION OF PLATES 1 AND 2

## PLATE 1

- Fig. 1. Photomicrograph ( $\times 800$ ) of a Bowie's preparation of pancreas of dog 220. This animal was not responsive to the eleven daily injections of anterior pituitary extract it received. A small islet composed of beta cells and surrounded by acinar cells containing large black zymogen granules, can be seen in the photograph. The fine dark granules seen in this islet are those of the beta cells. This is approximately a normal content of beta cell granules.
- Fig. 2. Photomicrograph ( $\times 800$ ) of a Bowie's preparation of pancreas of dog 221. This dog received eleven daily injections of the extract. The islet seen in the centre of the photograph reveals only a few fine dark-staining beta cell granules sprinkled through disorganized cytoplasm. The empty spaces are the result of hydropic change.
- Fig. 3. Photomicrograph ( $\times 800$ ) of a Bowie's preparation of pancreas of dog 217. This dog received eleven daily injections of the extract but in addition it received insulin. One alpha cell with its darker staining granule can be seen in the centre of the islet. The surrounding beta cells have retained approximately their normal granulation as is evident from the fine stippling seen in the photograph.
- Fig. 4. Photomicrograph ( $\times 800$ ) of a haematoxylin and eosin preparation of pancreas of dog 280. This animal received seven daily injections of the extract. The islet demonstrates hydropic degeneration.

## PLATE 2

- Fig. 5. Photomicrograph ( $\times 800$ ) of a Bowie's preparation of pancreas of dog 280. No beta cell granules can be seen in this islet, the cytoplasm of the beta cells is disorganized and hydropic.
- Fig. 6. Photomicrograph ( $\times 800$ ) of a Bowie's preparation of the pancreas of dog 241. This animal received seven daily injections of the extract plus insulin, but its diabetes could not be controlled with the latter. No beta cell granules are apparent in the islet shown in the photograph.
- Fig. 7. Photomicrograph ( $\times 800$ ) of a Bowie's preparation of pancreas of dog 279. This animal received seven daily injections of the extract plus insulin. The portion of the large islet illustrated in the photograph is composed chiefly of beta cells replete with granules.
- Fig. 8. Photomicrograph ( $\times 800$ ) of a haematoxylin and eosin preparation of pancreas of dog 217 (receiving A.P.E. plus insulin). A mitotic figure can be seen in the islet.
- Fig. 9. Photomicrograph ( $\times 800$ ) of a haematoxylin and eosin preparation of pancreas of dog 217. Two mitotic figures can be seen in this photograph. They are in acinar cells.

## ON THE INFLUENCE OF WEAK ELECTRIC CURRENTS AND ELECTRICALLY CHARGED SURFACES ON BLOOD COAGULATION

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SINCE Sir Charles Scudamore [1824] studied the effect of an electric current on the blood, only two papers have appeared on this subject. Stern [1916] made an experimental study on the influence of current on blood coagulation; Leiri [1934] made an interesting study from a purely theoretical viewpoint, without reporting any experiments. Stern used the method of Cannon & Mendenhall [1914] to register the coagulation time of blood. In this method the movements were recorded of a wire loop which just fitted into a very narrow tube filled with blood; Stern used this wire as the positive pole. His claims of a reduction of the coagulation time of blood due to current flow were found in the experiments described below not to be quite correct. Since with this technique only occurrences on, or very near to, the positive pole could be observed, his claims hold good only for the immediate surroundings of this pole. What happened in the bulk of the blood and on the negative pole escaped observation.

Neither Scudamore nor Stern used electrodes of chemically inert metals, such as platinum, etc. They did not investigate whether their results were due to what might be called non-specific coagulation of some proteins by the current, or to a true influence on the physiological process of blood coagulation. It was the purpose of the experiments described below to get some information on these lines, and a method was designed which allowed a rough quantitative comparison of the action of different electrodes and at the same time permitted distinction between what happened on the charged surfaces and in the bulk of the blood.

## METHOD

Since it was seen in preliminary experiments that the clotting process changed gradually on passing from the positive to the negative pole, the use of a mechanical device recording the clotting time was thought to be unsuitable, since this would only record what happens around one electrode or on one point between the poles. It was thought better to avoid the movements of the electrodes or the recording wires (as is necessary in the Cannon & Mendenhall technique), since the movements of charged surfaces would rhythmically alter the field and would complicate the interpretation of the results.

Two electrodes were fixed at a distance of 1.8 cm. from each other, and a neutral electrode of similar shape placed at the same distance from either. 10 c.c. blood was drawn from the veins of healthy individuals (mostly medical students) by means of a clean syringe and a stainless needle. The blood was mixed within the syringe by drawing in some air, and equal quantities were placed quickly into small Monax-glass basins of diameter 4 cm. The latter were well cleaned and dried by direct flaming. Immediately after the blood was placed into these vessels the electrodes were lowered into the blood of one of them, so that the poles reached nearly to the bottom of the vessel, the distance from the bottom being maintained in most cases at less, and never more, than 1 mm. The current was passed from the moment the electrodes were placed in the blood. The area of the electrodes was allowed to vary slightly, and the current was kept constant during the experiment. The electrodes were in most cases sheets of the respective metal, but also rods or thick wire were used (see Table 1). As far as possible an approximately equal area of the electrodes was in contact with the blood (c. 50 mm.<sup>2</sup>, except for platinum, where the area was c. 30 mm.<sup>2</sup>). The experiments were carried out with current densities ranging from  $5 \times 10^{-3}$  to  $10^{-2}$  amp./cm.<sup>2</sup> Both basins were left undisturbed for about 3 min. Then, by slightly tilting the control vessel, it was observed when the blood there was just clotted, and at this moment the electrodes were lifted out from the other vessel. All liquid on the electrodes was sucked off by means of a filter paper. The clot remaining on them was left to dry in the air. The dried clot was scraped off and weighed, and this weight was divided by the surface area of the metal which was previously in contact with the blood (clot: mg./mm.<sup>2</sup>). On those electrodes where there was obviously no clot there was, of course, always some dried blood left, after the application of the filter paper. The weight of this d

comparison with the weight of the clot that it was neglected. The probable error of the method was estimated by weighing the dried clot which appeared on ten electrodes of the same kind, and under the same conditions, and is thought to be about 25%.

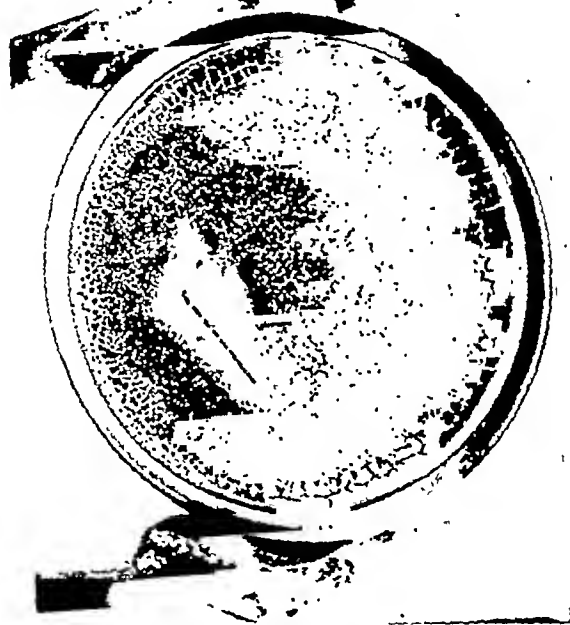


Fig. 1. The dotted line shows the position of the negative aluminium electrode, the other line that of the positive aluminium electrode. The blood was poured out from the vessel when clotting started. Delay of clotting on and around the negative (aluminium) pole.

When the blood in the control vessel (no current) was just clotted, the bulk of the blood in the vessel where the current was applied was always found to be still liquid. When the liquid portion was poured out the bottom of the vessel offered a very significant picture in many cases (Fig. 1). Since the blood clots first, as is well known, on the glass surface, any delay or acceleration of clotting on or around one electrode was easily observed by the picture which appeared on the bottom of these vessels. From Fig. 1 it can be seen that the blood around one electrode



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comparison with the weight of the clot that it was neglected. The probable error of the method was estimated by weighing the dried clot which appeared on ten electrodes of the same kind, and under the same conditions, and is thought to be about 25%.



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was still liquid and was poured out with the bulk of the blood, the area around this electrode therefore appears light. Where there is a layer of clotted blood it appears dark. If observed from the side, in most cases also a typical picture was observed (Fig. 2).

At *A* it can be seen that the layer of clotted blood is higher near the positive pole, and becomes gradually thinner towards and disappears totally in the area around the negative pole (aluminium electrodes).

Before use the surfaces of the electrodes were rubbed with rough emery cloth and finally polished with a smooth emery cloth and a rough linen. Thereafter they were brushed with soap powder and hot water, kept in running hot water for several minutes and, after rinsing with distilled water, dried between filter paper. Care was taken that the area which had to come in contact with the blood was not touched with the fingers and further, that no small particles of the filter paper adhered to them. Only metal pieces were used which had not been used previously as either poles. The reason for this precaution will be discussed later.

In using this very simple procedure the individual variations of blood clotting were thought to have been well excluded since the time during which the current was applied, and the moment at which the blood which was still liquid was poured out, was always determined by the clotting time of the same blood in the control vessel.

#### RESULTS: DELAY OR ACCELERATION OF BLOOD CLOTTING ON OR NEAR THE TWO POLES AND IN THE BULK OF THE BLOOD

The results are shown in Table 1. They can be divided into three groups. In the first two groups a definite and far reaching inhibition was observed on one of the two electrodes, namely at the negative electrode for metals of the first, at the positive electrode for metals of the second group. In group 3 no such inhibition could be observed, since there was clotting on both electrodes. But there was a little more clot on the positive than on the negative with some of the metals, and the reverse with other metals of this group. Both electrodes were of the

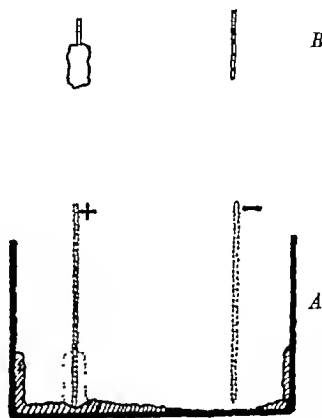


Fig. 2. The dotted lines show the position of the aluminium electrodes as they were before the blood was poured out. The shape of the clot on the same electrodes, after they have been lifted out, is shown at *B*.

same metal in each experiment. All the metals used were stated to be pure, except iron which was stated to contain 99.5% pure iron; the cobalt sample was stated to contain 92% pure cobalt, the rest being carbon and cobalt oxide.

TABLE 1

Electrodes	Clot: mg./mm. <sup>2</sup>			Remarks on groups
	-	-	Neutral	
Brass (rod)	0.106	0	0.049	1st group:
Cu (sheet)	0.101	0	0.040	Inhibition of clotting on and around cathode as shown in Fig. 1
Al (sheet)	0.128	0	0.051	
Zn (sheet)	0.256	0	0.063	
Duralumin (sheet)	0.098	0	0.058	
Sn (rod)	0.062	0	0.048	
Fe (wire)	0	0.125	0.048	2nd group:
Mn (sheet)	0	0.031	0.025	Inhibition as shown in Fig. 1, but around anode
Ni (sheet)	0	0.040	0.056	
Pb (sheet)	0.059	0.030	0.050	3rd group: No definite inhibition clot: - > -
Co (rod)	0.027	0.019	0.158	
Cd (rod)	0.058	0.034	0.028	
C (arc)	0.045	0.028	0.030	
Ag (rod)	0.041	0.015	0.020	
Pt (wire)	0.012	0.055	0.025	clot: - < -
Au (sheet)	0.021	0.042	0.027	
Mg (rod)	0.025	0.045	0.052	

The following result was common to all experiments quoted in Table 1. The bulk of the blood in the vessel where the current passed through, always clotted later than the blood in the control vessel. When a number of electrodes (Al. Pt, Zn, Ni) were immersed without applying a current, no such delay could be observed. The delay in clotting is therefore probably due to the current, whatever delay or acceleration takes place on or around the different electrodes.

#### EXPERIMENTS WITH BLOOD MADE INCOAGULABLE

Since Steinlein [1853] showed a coagulation of egg white on the positive pole of oxidizable metals (Cu, Zn, Fe, etc.) such effects on proteins have often been observed, and it appeared therefore of interest to see whether the currents as applied in the experiments described above did exert to some extent such an action; in other words, if it was or was not the physiological process of blood clotting which was affected.

Neither with Pt, Au, Al or Cu electrodes and with the currents applied as before could any visible coagulation of blood be observed when the blood was made incoagulable. As anticoagulants 1.2% potassium oxalate, 1.2% sodium citrate and heparin were used. The heparin sample was of approximately 80% the potency of the sodium salt of heparin,

prepared by Dr Jorpes of Stockholm, and was used so that the blood contained 0.1% of it. The current was allowed to pass for a longer time (12 min.) than in any of the experiments quoted in Table 1, through heparinized, oxalated or citrated blood and through serum, and there was never any obvious amount of coagulated substance nor any obvious difference in the amount of liquid attached to the electrodes after they were lifted out. Though it is probable that in the cases where Zn, Cu or Al were used a coagulating action on some proteins had taken place, the experiments showed that it was not of the same order as that observed with blood which could still coagulate normally. The effects shown in Table 1 are therefore believed to be due to a true influence on the process of blood clotting.

Experiments with recalcified oxalated blood gave results which were similar in principle to those obtained with freshly drawn blood, though they were much less pronounced, and the definite inhibition of clotting around the negative pole of aluminium and brass electrodes was not so evident as with freshly drawn blood.

#### THE CONTACT ANGLE OF BLOOD ON THE ELECTRODES

Since Bordet [1921] discovered that blood clotting is delayed on surfaces which are not wetted by water (e.g. paraffin) in comparison with surfaces which are wetted (e.g. glass), it appeared interesting to observe the contact angle at the blood-electrode-air junction, as an expression of the wettable or unwettable process. This was also thought important as a result of Lampert's [1931] considerations on the bearing of the surface tension of different substances in relation to blood clotting.

When the electrodes were immersed in the blood, the contact angles were very different on the two electrodes as shown in Fig. 3. This effect showed a curious hysteresis: when the electrodes were lifted out of the liquid again, so that the junction was formed on a surface which was previously in contact with the blood, the difference between the contact angles on the two electrodes disappeared and became on both similar to that which appeared previously (while moving downwards) on the negative pole only. When a dry pair of electrodes was immersed and fixed at the lowest point (i.e. without lifting them out while fixing) the difference of

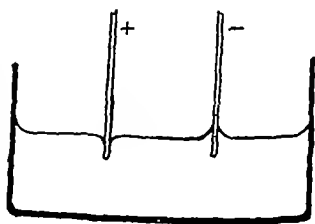


Fig. 3. Types of contact angle of blood on the two poles.

contact angle remained very pronounced. If then the current was switched off, no notable difference occurred. The previously positive and the previously negative pole continued to produce the different types of contact angle.

No measurements of the contact angles were carried out. It cannot be stated therefore if there was not (as might be expected) a small change of the contact angle after the current was switched off, a change which might well have escaped the rough direct observation. It can, however, be stated that an effect of the latter kind (*electrocapillary effect*) is certainly not mainly responsible for the two different angles, because of the persistence of the effect after the current was switched off.

The difference in the contact angles must be attributed therefore to the known changes in the surfaces of metals when they are used as anodes or cathodes. The 'anodic passivity' of metals, the formation of oxide films on the surface of noble metals, are well known examples for such changes (see Adam, 1938; Butler, 1939).

Taking these observations in conjunction with those of Bordet on the role of 'wetting' in blood coagulation one might anticipate an acceleration of blood clotting on the negative pole, because it becomes wettable and a delay on the positive pole, because it becomes distinctly unwettable.

In order to investigate whether that postulate holds good the following experiment was made. A number of aluminium sheets, hitherto not used as electrodes, were cleaned and polished and partly used as cathodes, partly as anodes, for 15 min. in a 1% NaCl solution ( $5 \times 10^{-2}$  amp./cm.<sup>2</sup>). Thereafter they were rinsed with distilled water and carefully dried in filter paper. These sheets were then immersed in freshly drawn blood and, without letting any current pass through, were left in the blood for such a time that in a control vessel the coagulation of the blood had just started, but was not yet complete. At this moment the aluminium sheets were lifted out and the dried clot per unit surface area determined as before. Immediately from their appearance one could see that on all sheets previously used as cathodes there was much more clot than on those which were previously used as anodes. The weights of the dried clots showed that about twice as much clot formed on the metals previously used as cathodes, as on those previously used as anodes. These experiments were carried out with aluminium, platinum and brass.

It should be noted that in the case of aluminium and brass the contrary effect (more clot on the anode than on cathode) is observed

when a current is applied. This adds weight to the view that the results obtained with the metals of this group (1st group, Table 1) are due to an action of the metal ions liberated by the current. The effect of the ions liberated by the current obviously more than counterbalance the effect of the difference of 'wetting' of the two poles. In the case of platinum, however, about the same result is obtained either with a current or without a current, if in the latter case one piece of platinum was previously used as a cathode, the other as an anode. This suggests that the effect obtained with a current and platinum electrodes is mainly, if not wholly, due to the change of the platinum into wettable and unwettable surfaces on the respective electrodes. It is therefore likely that many of the metals of group 3 have no action on the blood coagulation, and the difference observed on the two poles is due to the change of the respective three surface tensions involved in the contact angle, as represented in the Dupré equation. The difference in 'wetting' of 'anodized' or 'cathodized' metals is very obvious if a drop of water is placed on two such sheets of the same metal. When the sheets are tilted carefully, keeping both pieces at the same, slowly increasing, angle, the drop drains away from the cathodized sheet, while still staying on the anodized piece.

These experiments show that the surface of many, if not all, metals can be changed (by using them as anodes and cathodes respectively) into surfaces where the coagulation of blood is relatively delayed and accelerated respectively.

#### DISCUSSION

The fact, regularly found with whatever electrodes were used, namely, that the bulk of the blood, where the current passed through, clotted later than the control blood, is in contradiction to that found by Scudamore & Stern. The acceleration found by Scudamore [1824] was probably due to the heat which must have been produced by the strong currents applied by this author, while Stern's [1916] technique, as already mentioned, only recorded what was happening on or very near the positive pole and his claim for the acceleration cannot be extended to the bulk of the blood. No explanation can be offered for the delay in clotting of the bulk of the blood, because no special experiments were made to investigate the mechanism of this phenomenon, but it might tentatively be suggested that the reason might be found to be connected with the migration (due to the current) of molecules which are involved in the clotting process.

The results obtained with the chemically inert metals of group 3 seem to be explained, at least to a great extent, by the changes in the

contact angles and by the fact that the current quickly alters the metal surfaces so that they become wettable or unwettable on the respective poles. The very pronounced delay of blood clotting on the negative pole of the metals of group 1, and that the exact reverse was found with the metals of group 2, is not easily explainable. Experiments with these and other metals are being carried out in order to find a common characteristic of the metals forming group 2, with all of which there is pronounced inhibition of clotting in the region of the anode.

### SUMMARY

A weak direct current was applied to freshly drawn blood. The results obtained with different electrodes could be divided into three groups, according as delay, inhibition or acceleration of blood clotting was observed on the two poles. Since the same electrodes with similar currents had no very obvious coagulating effect on proteins of heparinized blood, it is inferred that in the former experiments there was a true influence on the blood-clotting process. The clotting of the blood where the current passed through is always delayed; whatever delay or acceleration of the clotting takes place on or around the different electrodes.

Some results were explained by observations of the contact angles of blood on the two poles and led to experiments which showed that on a metal surface, which was previously used as cathode, blood clotting is relatively accelerated, while it is delayed on the same metal when it was previously used as anode.

I should like to thank Prof. H. P. Gilding for his helpful advice and for the loan of apparatus. I am also grateful to the Birmingham Research Laboratory of the Mond Nickel Company Ltd. and to W. Canning and Company for presenting me with samples of various metals.

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VARIATIONS IN THE WATER, FAT, GLYCOGEN AND  
IODINE OF THE FLESH OF OYSTERS (*OSTREA*  
*VIRGINICA*) DURING HIBERNATION AND  
STORAGE AT 4° C.<sup>1</sup>

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*OSTREA VIRGINICA* is the common oyster of the north Atlantic seaboard of Canada and the U.S.A. It spawns in the summer, 'fattens' in the autumn, 'hibernates' in the winter, and becomes active with the production of spawn in the spring. The oyster beds are farmed in the 'R' months of September to April, because during these months the oysters are fattest, spawning is not interrupted, and the low environmental temperature favours keeping. The oysters are then marketed in the shell, or canned, or quick-frozen or shucked (i.e. removed from the shell). Marketed live oysters are kept at a low temperature, usually packed in ice, until ready for use, and under good conditions will keep for several months.

The object of the present investigation was to study changes in the composition of oysters during the hibernating period when stored at a temperature of 4° C. over a period of 3 months, which we have found to be the maximal time they can be kept at this temperature before the onset of putrefaction. The oysters used were obtained from a lagoon off St George's Bay near Tracadie on the north-east coast of Nova Scotia. It is probable that the results obtained on oysters from this locality would be similar to those on oysters from adjacent localities, because Coulson [1934] observed no significant variation in the chemical composition of oysters collected at various points along the Atlantic coast of the U.S.A. Masumoto, Masumoto & Hibino [1934] added the information that seasonal changes in the amount of fat, protein and glycogen of oysters may occur earlier in different oyster beds, a change which might well be expected.

<sup>1</sup> This paper was presented before the Annual Meeting of the Canadian Physiological Society, Montebello, Quebec, October, 1941.

## METHODS

*Storage.* The oysters were shipped to us in the shell and without washing. When oysters are washed with fresh water, they rapidly lose part of their constituents such as ash [Smith, 1919], iodine [Coulson, 1934] and chlorides [Krogh, 1938]. The shipment reached this laboratory in a large box weighing 175 lb., and it is possible that the composition of the oysters at the bottom of the box had altered, since excessive weight over a long period has been stated to speed up their gaseous metabolism [Nozawa, 1929]. When the box was opened the oysters were mixed before using.

The shipment was stored from October to January in a room at 4° C. in which the relative humidity was always close to 100%. At this temperature the shells do not open, and the gills and palps become coated with a mucus-like material which prevents or diminishes external respiration [Pease, 1932], but metabolism continues, as evidenced by the accumulation of reducing substances and of ammonia and amino acids [Clark & Almy, 1917], which are the chief end-products of the inefficient protein metabolism of the oyster [Spitzer, 1937]. Oxygen consumption is low [Dušková, 1931], but under anaerobic conditions molluscs produce carbon dioxide by anaerobic respiration of glycogen and acids are neutralized by calcium carbonate [Collip, 1921], which makes up 94% of the shell [Pease, 1932], the chalk being etched out by the carbonated water to form calcium bicarbonate in the mantle fluid [Kühtide, 1938].

*Analyses.* At intervals of 2-4 weeks for a period of 3 months, thirty or more oysters were removed for analysis. The shell was cracked, side-knifed, stabbed and the flesh lifted out. Glycogen was immediately estimated by Pflüger's method as modified by Good, Kramer & Somogyi [1933]. The flesh of the remaining oysters was individually dried at 90° C., the wet and dry weights ascertained and the dried substance analysed for fatty substances and iodine by methods previously used in this laboratory [Boyd, 1938; Boyd & Clark, 1940].

The sterols were determined by digitonin precipitation before and after hydrolysis, giving values for total and free sterol and, by difference, esterified sterol. Cholesterol is precipitated by digitonin from alcohol-acetone as is well known, and Sperry & Bergmann [1937] have reported that ostreasterol is precipitated to about 70%.

## RESULTS

*Body water.* The water content of oysters estimated immediately after removal from the shell remained unchanged over a period of 1 month. Minimal, maximal and mean values are given in Table 1 along with the

TABLE 1. Water content (g./100 g. wet weight) of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
3	81.4	86.5	83.2	0.53
5	79.9	85.3	83.6	0.56
7	79.0	82.4	80.7	0.39
9	77.5	84.3	81.5	0.63
13	78.2	83.7	81.1	0.59

standard errors of the means. The mean value at the end of 3 weeks' storage was 83.2% water, and after 5 weeks' storage it was 83.6%. There was little variation in the averaged results, Pearson's coefficient of variation being 1.9 and 2.1 respectively, which are low values for biological material. This small variation is all the more remarkable when it is recalled that the oysters simply lay in the shell in a box which was partially open in the refrigerator room, that the outside of the shells was almost dry and indicated that evaporation was active, and that the only watery material bathing the oyster flesh was the mantle fluid. The data further indicate that the posterior catch muscle of the oyster is an efficient mechanism for fixing the position of the valves over a long period of time. Considerable variation has been reported by Shannon [1913] in the water content of shucked oysters, his values running from 77 to 97% of total body weight. The mean values reported in Table 1 are slightly higher than other values in the literature for oysters in the shell, such as those reported from Long Island, N.Y. [Pease, 1932], and the Pacific coast of the U.S.A. [Albrecht, 1920].

Between the 5th and 7th weeks of storage at 4° C., the water content decreased a slight but statistically significant amount, namely, 2.9%, a mean difference which had a standard error of 0.68, indicating that the odds were 1 in 370 that it was due to chance alone [Hill, 1939]. After 9 and 13 weeks' storage, the water content remained significantly lower but did not progressively decline. Had the loss of water after 5-7 weeks been due to evaporation or escape of water through imperfectly sealed mantle tentacles, one would have expected a progressive decline in body water. The results thus suggest that some change in metabolism occurred at this time, a change which was accompanied by

a decline in body water and a shift of water into the mantle cavity. This suggestion is borne out by further analyses as described below.

*Body fat.* Values for the total fatty substances of oyster flesh in the shell have been summarized in Table 2. These varied from 4.7 to 8.9% of the dry weight with an average of 6.8%, and there was no significant change during the 3 months of storage. A corresponding absence of

TABLE 2. Total body fat (mg./100 g. dry weight) of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
3	5721	7738	6660	208
5	4659	8486	5923	325
7	5512	7837	6849	201
9	5950	8849	7031	228
13	6171	8483	7012	263

seasonal changes during storage was noted by Malcolm [1928] on Stewart Island oysters of New Zealand. When oysters and other molluscs function as females, they have been reported to contain more fat [Masumoto, Masumoto & Hibino, 1932; Timon-David & Ceresola, 1935], a finding which may reflect the tendency of oysters to change from males to females under the influence of good nourishment. We are unable to confirm this statement, since no determinations were made of the sex of our animals. The values for 'fat' reported by Pease [1932] on Long Island oysters were higher than those shown in Table 2, but our oxidative procedure for estimating total body fat gives more accurate values than those which can be obtained by weighing the ether extractives as used by Pease.

*Body sterols.* The oyster contains a number of sterols, some of which are related to phytosterols and some to zoosterols. It contains cholesterol [Bergmann, 1934a], ostreasterol, which is an isomer of stigmasterol [Bergmann, 1934b], small amounts of stigmasterol [Bergmann, 1937], 'conchasterol' [Tsujimoto & Koyanagi, 1934] and ergosterol or ergosterol-like sterols [Gillam & Heilbron, 1936]. Of these, ostreasterol and cholesterol are the only ones which seem to be present in large amounts. Values for the sterol fractions are given in Table 3 with all sterols calculated as cholesterol.

There were no changes in the total sterol content during storage which were considered significant. The values for sterol ester and free sterol showed apparently significant variations at times, but it was noted that when free sterol had decreased, ester had increased and vice versa, and there was no regular or consistent trend. It is possible that these

TABLE 3. Sterol content (mg./100 g. dry weight) of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
Total sterol				
3	532	934	670	39
5	624	890	738	30
7	404	922	664	62
9	453	655	548	20
13	486	797	628	26
Sterol ester				
3	61	685	323	59
5	72	395	225	34
7	70	382	249	37
9	102	365	260	27
13	72	366	194	25
Free sterol				
3	175	624	347	54
5	397	590	513	17
7	293	590	415	35
9	221	357	288	11
13	275	642	434	35

apparent variations were due to variations in technique resulting in more or less hydrolysis of esterified sterols during the estimation of free cholesterol which, in the procedure used, was precipitated by digitonin and by concentrating with heat a mixture of free and esterified sterols in acetone. Excessive heat under these conditions will hydrolyse cholesterol esters, and ostreasterol esters may be still more readily hydrolysed. Until more data are available on these several points, it would be inadvisable to conclude that variation occurred in the sterol fractions during storage.

*Body neutral fat or residual fatty acids.* In oxidative micromethods, the term neutral fat is applied to the residual fatty acids which remain after subtracting from the total fatty acids the sum of the phospholipin fatty acids and sterol ester fatty acids and expressing the difference as triglyceride fat. In mammalian blood and tissues these assumptions are reasonably correct, but in the oyster it would be more conservative to refer to this fraction as residual fatty acids. Further, the values for oyster neutral fat are undoubtedly relatively, rather than absolutely, correct, because the various factors used in their computation were worked out for mammalian blood fats and depend upon the composition of these fats, a composition which is undoubtedly different in the oyster from that in mammalian blood. As shown in Table 4, the 'neutral fat' content of oysters remained constant during storage at a value which averaged 4.3% of the dry weight.

TABLE 4. Neutral fat content (mg./100 g. dry weight) of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
3	2740	5890	4500	305
5	2950	5750	3950	253
7	2740	5900	4633	331
9	3480	6200	4207	274
13	3780	5800	4333	227

*Body fatty acids.* The fatty acids isolated from oyster flesh were liquid at room temperature, a fact which has been noted before [Pease, 1932]. The total amount of fatty acids, calculated as oleic acid, remained relatively constant during storage at an average value of 5.3% of the dry weight. These results are shown in Table 5.

TABLE 5. Total fatty acid content (mg./100 g. dry weight) of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
3	4230	6270	5340	192
5	3590	6550	4629	292
7	4050	6330	5502	239
9	4660	7390	5576	235
13	5000	6880	5662	200

*Body phospholipin.* No significant changes occurred in the total amount of any of the above fatty substances during storage. There was, however, a significant increase in the amount of phospholipin after 2 months' storage at 4° C. Values given in Table 6 are for acetone-

TABLE 6. Phospholipin content (mg./100 g. dry weight) of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
3	720	2240	1310	141
5	686	1690	1076	120
7	835	1780	1386	103
9	1620	2400	2102	95
13	1540	2280	1971	93

insoluble lipins calculated as lecithin-cephalin, and after 2 months' storage there may be seen to be a significant increase of some 65% in the amount of this lipin present in oyster flesh. From the data presented in Tables 1-6, it is obvious that some of the additional phospholipin may have been synthesized from neutral fat, some from sterol esters, and perhaps some from non-fatty sources, but the data do not permit of a definite conclusion as to the source of the new phospholipin.

*Body glycogen.* Mollusc flesh is rich in glycogen, and that of oysters is especially so. Oyster glycogen may not be identical with mammalian glycogen as Hayashi [1932] states that it is less easily hydrolysed than mammalian glycogen, and a purified glycogen prepared by McDowell [1927] was found to contain less phosphorus than a correspondingly purified mammalian glycogen. The glycogen content of oysters has been reported to be highest just before hibernation, with slowly decreasing values during hibernation and a rapid decrease during activity and spawning [Malcolm, 1928; Masumoto *et al.* 1934; Tully, 1936]. Glycogen analyses are summarized in Table 7. After 2 months' storage, there was

TABLE 7. Glycogen content (g./100 g. dry weight)  
of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
7	9.73	12.9	10.9	0.66
13	5.00	7.18	6.18	0.32
15	5.91	9.55	7.64	1.73

a fall in glycogen coincident with the decrease in body water and the increase in phospholipin, which is further evidence that some major change in body metabolism takes place at this time.

*Body iodine.* After 2 months' storage there also occurred a decrease in the iodine content of oyster flesh as shown in Table 8. Before this fall, body total iodine averaged 433  $\mu\text{g.}/100\text{ g.}$  body weight of dry tissue,

TABLE 8. Iodine content ( $\mu\text{g.}/100\text{ g.}$  dry weight)  
of Nova Scotia oysters (*O. virginica*)

Weeks' storage	Minimal value	Maximal value	Mean	Standard error
3	266	552	406	30
5	358	672	473	30
7	372	534	420	15
9	226	592	366	30
13	242	498	340	24

a value similar to that of Coulson [1934] for oysters obtained from the Atlantic and Gulf areas of the U.S.A. During the last month of storage the average iodine content fell to 353  $\mu\text{g.}$ , a decrease which was calculated to be statistically significant. These results indicate that at this time iodine diffuses from the flesh into the mantle fluid, the latter not being included in any of the material analysed. Thus, associated with the changes in body water, body phospholipin and body glycogen, there also occurred changes in body iodine after 2 months' storage at 4°C.

## SUMMARY

1. An analysis of Nova Scotia oysters, *Ostrea virginica*, collected in October 1940, and stored in the shell at 4° C., revealed the following mean values per 100 g. dried flesh (except in the case of water content which is expressed as per 100 g. wet weight): water, 83.2 g.; total fat, 6.66 g.; neutral fat, 4.50 g.; total fatty acids, 5.34 g.; total sterols, 0.67 g.; sterol esters, 0.32 g.; free sterols, 0.35 g.; phospholipin, 1.31 g.; glycogen, 10.99 g.; iodine, 0.34 mg.

2. The oysters were stored in the shell for a period of 3 months at 4° C. During the first 2 months' storage no appreciable change took place in the amount of any constituents. In the last month there was a significant decrease in water of 2.5%, in glycogen of 37%, in iodine of 18% and an increase in phospholipin of 65%.

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## MINERAL METABOLISM OF HEALTHY ADULTS ON WHITE AND BROWN BREAD DIETARIES

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APART from a few numerically unimportant races, such as the Esquimaux and Masai, humanity relies extensively for its nourishment upon starches and proteins of vegetable origin. Exceptionally, these may be furnished by the potato, as in Tristan da Cunha, or by kassava, as in certain parts of India and Africa, but cereals constitute the important supply for the great majority of mankind. There are at least seven used for human consumption in different parts of the world—wheat, rice, maize, millet, rye, barley and oats. Each of these may be processed in many different ways. There must therefore be problems galore about the merits and demerits of cereals; and yet, oddly enough, they have been relatively little explored. One of them was propounded by Mellanby [1925] when he discovered that there was some connexion between the severity of the rickets developed by a growing dog and the type of cereal upon which it had been fed. Oatmeal was highly rachitogenic, whereas white wheat flour was relatively innocuous. At the time, and subsequently, the general importance of this discovery was missed, mainly perhaps because the experiments centred upon the production of rickets in growing animals and its cure by vitamin D. The last was therefore seized upon as the important variable. Until very recently it would have been safe to say that in spite of a great deal of highly suggestive work, vitamin D has remained the centre of interest in calcium metabolism, and the nutritional properties of cereals have been regarded as beyond practical control.

Quite apart from Mellanby's work on rickets, our knowledge of cereal chemistry has undergone considerable expansion in the last 20 years, and striking differences have been discovered between the composition of different cereals, and also between the chemistry of the whole

grain and most of the milled flours prepared therefrom. All these differences have nutritional significance. It has frequently been demonstrated, for instance, that white flour contains less phosphorus, iron, magnesium, vitamin E and the B group of vitamins than an equal weight of the whole wheat berry. It also contains less calcium and probably a less nutritious assortment of amino acids. It has, moreover, none of the laxative properties of wholemeal, owing to the fact that it contains practically no bran. The advocates of wholemeal flour have not been slow to seize upon these findings and to exploit them to the best of their ability, but they have said very little about the fact that a large part of the phosphorus in wholemeal flour is present as phytic acid. This compound, in which one molecule of inositol is linked with six phosphoric acid radicles, forms insoluble calcium, magnesium and iron salts and it is not hydrolysed by any of the enzymes produced by the human digestive tract. When, therefore, work which was carried out in this department in the first six months of the war, demonstrated the valuable nutritive properties of a wheatmeal containing 92% of the original grain, but at the same time suggested that the consumption of large amounts of it might be unfavourable to calcium absorption, a comprehensive set of experiments was planned to try to clarify some of these interesting and practical aspects of cereal chemistry and human nutrition. The development of our knowledge of phytic acid has been traced in a recent editorial article in *Nature* [1941].

The experiments now to be described were designed to solve the following problems:

- (1) Do men and women absorb calcium less freely from brown than from white bread dietaries?
- (2) If this should prove to be the case
  - (a) Is phytic acid the noxious agent, and if so has it any effect on other metals?
  - (b) Will vitamin D restore the calcium absorptions and balances to their white bread levels?
  - (c) Will the fortification of wheatmeal flour with calcium salts overcome its bad effects on the absorption of this element?

And if so

- ( $\alpha$ ) What is the best salt to use?
- ( $\beta$ ) How much should be added?

## THE METHODS AND SUBJECTS

The work now to be described has been carried out by 'balance' experiments on normal healthy human subjects. Each experiment, i.e. each set of conditions, has lasted for at least 14 and usually 21 days. Some have lasted for 28 days. The control experiments have been as long as the experimental ones, and have been repeated if and when there seemed any need to do so. The subjects have been given at least 3 and often 7 preliminary days in which to adjust their metabolism to a change of conditions, and the experimental diet has never been stopped till the last collection of faeces has been made. Each experiment has been subdivided into two, three or four analytical periods of 1 week in length. If an unexpected answer has been obtained for any one set of analytical results, and such errors as sampling have been excluded, the question has always been raised as to whether this represents a genuine irregularity of metabolism or a technical error in the collection of the faeces. It has been found very helpful in this dilemma to have simultaneous balances of several minerals, and this procedure may be recommended to others. If, for example, the calcium balance of the first week of an experiment is unexpectedly negative but the magnesium balance is exact, and the iron balance also, then the result must be regarded as due to a metabolic cause if contamination of the excreta can be excluded. But if the faecal magnesium is also very high and the iron balance grossly negative, then the probability is that the faecal marking of this week has been incorrect, and it may be legitimate to disregard this set of results if two others are available.

*The subjects*

There have been four men and four women in each experiment. Their initials, ages, sex, and some further information are given in Table 1.

*The dietary organization*

At first all meals were served in a large room in the Department of Medicine, and the subjects always partook of them there till they were thoroughly versed in the routine. Owing to the prolonged nature of the experiments and of the whole study, it became apparent after some time that most of the subjects would be very much happier if they were allowed some measure of freedom, and accordingly each was encouraged to take one evening meal during the week at home. This meant taking away weighed amounts of prepared food, or cooking and weighing the food at home and bringing back duplicate portions for analysis. Both

TABLE 1

Initials	Sex	Age years	Weight lb.	Notes
E. B.	M.	31	152	A Spanish doctor with experience of research. Had the most regular metabolism of all the subjects. Readily absorbed calcium
C. B.	M.	29	176	An Antarctic explorer. Unfortunately had to leave the party after the first two experiments to take up work in Palestine
N. K.	M.	25	150	A botanist and spectrographist. Absorbed calcium less readily than some, and was generally in negative calcium balance
R. M.	M.	41	135	Took more physical exercise than the other subjects. His power to absorb calcium declined steadily through the 9 months of observation
P. S.	M.	32	176	A Czech surgeon. Replaced C. B. in the experimental party
B. A.	F.	26	117	Dietician, took charge of all the catering and cooking
K. B.	F.	27	118	Zoologist, wife of C. B. Accompanied her husband to Palestine. Carried out the first two experiments only
A. M.	F.	26	164	A biologist. Calcium metabolism most consistent in spite of some irregular dietary practices partly due to severe dysmenorrhoea
E. W.	F.	33	124	Tended to gain weight throughout the experiment. Showed a progressive deterioration in calcium absorption similar to R. M.'s
P. W.	F.	25	142	Dietician employed at Addenbrooke's Hospital. Replaced K. B. Had a less regular absorption of calcium than the other subjects

*Note.* The experiments were arranged without reference to the women's menstrual periods, which appeared to make no difference to their absorption and metabolism of calcium, magnesium or other minerals investigated.

were troublesome, but for most people the sense of escape made it well worth while. In the last few months the whole party took their evening meals home with them on Sundays.

In a prolonged study of this kind it is theoretically desirable that the basal diet eaten in all the experiments, the results of which are to be compared, should be exactly the same. This was an impossible goal owing to the limitations of war-time catering, but arrangements were made to maintain a roughly uniform dietary throughout the whole series of experiments, and when this was for some reason overborne, efforts were made to find out whether the change had had any demonstrable effect upon the metabolism of the subjects. A large proportion of each person's diet was automatically fixed, for it was planned in advance that flour should always furnish 40-50% of the total calories. The flour, which was obtained from Foster Mills Ltd., Cambridge, was taken partly as bread, and partly as puddings, cakes and pastry. The amount of flour eaten was added up at the end of each week, and each person did his best to keep his flour

consumption about the same from week to week. This was a surprisingly easy task, as Table 2 shows, although naturally some subjects were a good deal better at it than others. The remainder of the diet was as liberal as official rationing would allow, except that cheese was forbidden,

TABLE 2. Variations in the consumption of flour from week to week

Subject	Exp. 2 (August)			Exp. 7 (Feb., March)		
	Week A	Week B	Week C	Week A	Week B	Week C
	g.	g.	g.	g.	g.	g.
E. B.	2170	2124	2546	2389	2405	246
C. B.	2826	2812	2651	—	—	—
N. K.	3168	3463	3217	3616	3561	3487
R. M.	2736	2999	2808	2882	2846	2973
P. S.	—	—	—	2350	2280	2501
B. A.	2153	2401	2358	1820	1865	1770
K. B.	2114	2111	2034	—	—	—
A. M.	2157	2034	1980	2114	2463	2359
E. W.	2058	2103	1994	2048	2065	2087
R. W.	—	—	—	1955	1842	1980

and milk was restricted. The quantities of the various foods eaten were left to individual choice, but personal tastes and the repetitive tendency of the meals saw to it that there was not much variation from one week or from one month to another. Occasionally, someone was observed to be eating such large quantities of a particular food that there was a risk of his calcium intake becoming too high. He was then advised to reduce his intake, or he was given less milk. This applied particularly to R. M.'s green vegetables.

To assist in maintaining a uniform diet, enough fruit was bottled and enough jam was made to last throughout the series of experiments. Enough marmalade for the whole study was very kindly presented to us by Messrs Chivers Ltd. There was a limited allowance of fruit and jam containing seeds, since these are known to have phytic acid in them. There was also a regular issue of other fruits, but this was probably not very important since the experiences of the fresh-fruit season indicated that variations in the consumption of seedless fruits did not upset the balances (see Table 3). The vegetables eaten varied to some extent with the season, but legumes were eaten sparingly, since they have a certain amount of phytic acid in them. Spinach and rhubarb were avoided and any other plants known to contain oxalic acid, since this has been shown to precipitate calcium in the intestine and prevent its absorption [Fairbanks & Mitchell, 1938; Kohman, 1939; Logan, 1940]. McLaughlin's [1927] human experiments showed this also, but oddly enough the author interpreted her results in the opposite sense.

The first three experiments took place during July, August and September, and there were considerable variations in the consumption of raw plums, greengages, pears and damsons as these fruits became first plentiful and then scarce. It was feared, for a time, that these supposedly laxative fruits might upset the experiments, but inspection of the weekly results showed that variations in their consumption made so little difference to the output of faeces or to the calcium balances, that it was decided not to curtail them. Some of the data upon which this conclusion was based are given in Table 3. The variations, if any,

TABLE 3. Effect of variations in the consumption of fresh fruit upon the weight of the faeces and upon the metabolism of calcium

Subject	Week	Fresh fruit g./day	Faeces (wet weight) g./day	Calcium absorbed mg./day	Calcium balance mg./day
R. M.	1a	29	255	240	+14
	1c	160	268	203	-10
E. B.	1a	10	179	265	+27
	1c	147	136	274	+60
E. W.	1a	41	63	94	-17
	1c	250	76	111	-20
K. B.	1a	44	124	126	-34
	1c	177	143	180	+20

produced by alterations in the intake of fruit are quite trifling when compared with the differences produced by a change from white to brown bread, or by other changes which have deliberately been made.

All food was weighed on Hanson spring balances, on which readings could be made quite accurately to a gram. The bread was baked daily according to specification by Mr and Mrs Wilkin of Barrington, Cambs. Each person had a weighed '1 lb.' loaf at the beginning of the day, and was expected to eat the whole of it during the 24 hr. The men usually required a further half loaf to make up their day's quota. The amount eaten by each subject was totted up at the end of the day and  $\frac{1}{10}$ th as much set aside from the same baking for analysis. All other foods except butter and jam were weighed at the table as they were served, and the result entered at once in a book kept for the purpose. Waste on plates, such as stones of fruit, was weighed, and its weight deducted from the first weighing. The weight of bread crumbs was disregarded, as it had been found by experiment to be negligible.

After each meal  $\frac{1}{10}$ th as much as each person had eaten was set aside in an aluminium bowl, which was kept closely covered and which was large enough to hold one week's aliquots for analysis. Foods such as raw apples and meat were cut up finely with stainless steel knives before

the  $\frac{1}{2}$ th portions were weighed. Butter and jam were weighed out for each person at the beginning of every week, and any left uneaten at the end of the week was weighed. Then  $\frac{1}{2}$ th of the amounts eaten were added to the bowl of food.

This method of sampling may be very accurate provided certain precautions are taken in the preparation of the food. Briefly, the principle to be adopted is either to serve and weigh each ingredient of a meal separately, or to make a sufficiently intimate mixture of the various components, so that an average helping is likely to be a representative sample of the whole dish. Sauces should be served and weighed as such, and not poured over food. Pastry is better to be made and weighed apart from the pie to which it gastronomically belongs. Green vegetables must be cut up before they are served so that each person, and his duplicate portion, receive a uniform proportion of inside and outside leaves, since these contain very different amounts of calcium.

Only glass-distilled water or soft artesian-well water was used for cooking and only glass-distilled water for drinking. Tea and coffee were measured in beakers marked with two rings. The volume to the lower was half the volume to the upper, and the latter was about a cupful. Half the volume measured to be drunk was set aside at once for analysis. Distilled water was not duplicated for analysis. Each person's ration of milk was measured out daily with a pipette into his or her personal milk vessel, and at the same time  $\frac{1}{10}$ th of the quantity was set aside in a bottle containing toluene. The orders were that all milk measured out must be consumed, and also the washings of the vessel. R. M. received 100 c.c. of milk a day, the others 150 c.c.

### *The analytical arrangements*

As already described, each experiment was divided into weekly periods. At the end of these, each person's 'wet' food, which had been collected in the aluminium bowl, was weighed and pulped up to a uniform paste in a large mortar. His bread, which had been kept separately in a large beaker, was dried at 100° C., and then weighed and ground to a powder. His preserved drinks, themselves half the originals, were measured and  $\frac{1}{2}$ th of them added to his milk which, as already stated, was  $\frac{1}{10}$ th the volume actually consumed.  $\frac{1}{100}$ th to  $\frac{1}{200}$ th of the volume of the fluid sustenances taken by each person was then measured into each of two silica crucibles (max. external diameter 6.7 cm.) and the contents dried at 100° C. Similar aliquots of the wet mixed food were added and dried, and lastly the appropriate aliquots of the dried ground bread, so that the crucibles finally contained the same fraction of the wet food,

fluids and bread consumed by the person in question during the week. By the same procedure  $\frac{1}{10}$ th of the week's fluids, wet food, and bread were collected in a 1 lb. jam jar and dried for permanent preservation. There was always some frothing while these foods dried off, and the pots required a little attention. The crucibles were next placed in an electric furnace [Haynes & McCance, 1939] and the contents ashed at about  $450^{\circ}\text{C}$ .

The faeces of each subject were collected throughout each week in a counterpoised glass bowl covered, when not in use, by a dinner plate. The periods were marked by carmine, taken before breakfast on the first day of each week. If one can believe the literature, 0.1 g. of carmine has generally been used for this purpose, but experience in this laboratory suggests that this is not nearly enough, and about 0.7 g. has always been employed. This was given in five capsules, and a similar one added to the duplicate food bowl to be incorporated in the analysis. At the end of the week the wet faeces were weighed, distilled water was added (if necessary) and about  $\frac{1}{20}$ th to  $\frac{1}{10}$ th of their weight of concentrated Analar  $\text{HCl}$ . They were then made into a uniform paste by pulping with a glass rod flattened at the end. The paste was weighed, and usually left overnight. It was then reweighed as a check on the original weighing and possible evaporation, stirred up once more and two samples of 50 g. taken from it and dried in silica crucibles. A further portion of the paste was preserved in a Forster fruit jar in case of analytical accidents or uncertainties, and the counterpoised glass bowl washed out. After the crucibles had been kept for 16 hr. at  $100^{\circ}\text{C}$ ., 5 c.c. of concentrated Analar  $\text{HNO}_3$  were added (1 c.c. at a time) to the hot contents, and the crucibles returned to the oven for a few hours. Some frothing sometimes took place during these manipulations, but was usually quite easy to control by tapping the crucibles gently on a cork or on the bench. The contents of the crucibles were ashed at  $450^{\circ}\text{C}$ .

The weekly urines were collected under toluene and  $\frac{1}{10}$ th retained for analysis. 100–200 c.c. were taken for analysis and dried off in silica crucibles, to which 1 c.c. of concentrated Analar  $\text{HNO}_3$  had been added. The ashing was carried out at  $450^{\circ}\text{C}$ .

The ashes of the foods and faeces were treated with 5 c.c. of concentrated Analar  $\text{HCl}$ , and taken down just to dryness on a sandbath. Then a further 5 c.c. of concentrated  $\text{HCl}$  were added, a watch glass placed over the crucible, and the contents heated to boiling over a small flame. The inside of the watch glass was washed into the crucible with 10 c.c. distilled water; the contents were again boiled, and filtered through a Whatman paper no. 41 into a 100 c.c. graduated flask. The



crucible was washed with four more additions of 10 c.c. of water, the washings being boiled each time before they were filtered. Any residual carbon was then transferred to the filter paper and repeatedly washed with boiling distilled water until the total volume was nearly 100 c.c. When cold, the solution was made up to volume.

The urine ashes were treated with 5 c.c. of concentrated Analar HCl and the contents of the crucible boiled. 10 c.c. of water were added, the mixture boiled and the extract filtered into a graduated 50 c.c. flask. Four subsequent extractions were made with 5 c.c. of water each time as described above, and the volume finally made up to 50 c.c.

The calcium, magnesium and other bases were determined as described by McCance, Widdowson & Shackleton [1936]. Phosphorus was determined in the unashed urines by Briggs's method [1922]. This element cannot be determined in foods by applying Briggs's method to the HCl extracts of a dry ash [McCance & Shipp, 1933], but it has been found practicable to use this method for faeces. A measured amount, usually 4 c.c., of the ashed extracts was heated for an hour at 100° C. to convert any remaining pyro- to ortho-phosphate, and was then washed out to 100 c.c. Briggs's method was applied to an aliquot of this. The phosphorus in foods was determined as described by McCance *et al.* [1936], on 0.5 g. of the ground-up samples of the dried mixed food which had been set aside week by week.

Two samples of all foods, faeces and urines were taken in parallel to be ashed so that duplicate extracts of each were obtained. A single chemical determination was made on each extract, and repeated if the agreement was not perfectly satisfactory. The procedure described has given excellent duplicates for calcium, magnesium, iron and potassium in faeces, and the same may be said of urines, although these contain very little iron. The calcium, magnesium and potassium duplicates in food have also been excellent, but the iron duplicates have been less perfect, and this is thought to have been due to the difficulty of obtaining a uniform distribution of meat, which is rich in iron, through the mass of food which had to be sampled for analysis. The differences were certainly not analytical in origin and were unlikely to have been due to contaminations.

## RESULTS

### *The composition and properties of 69 and 92% flours*

92% flour is generally recognized to contain somewhat less starch and more moisture, protein, fat, fibre, vitamins and minerals than 69% flour. The present flours conformed closely to type. The 'brown' contained

61.5% starch, 15% water, 2.45% nitrogen and 3.06% fat; the 'white' 67.5% starch, 13% water, 2.12% nitrogen and 1.37% fat. The iron in the 92% flour amounted to 3.55 mg./100 g. as against 1.41 mg. in the 69%. There was also more lithium, boron, manganese and cobalt in the 92% flour [Kent & McCance, 1941*a*, *b*]. The minerals of direct importance to the present investigation are calcium, magnesium, potassium, total and phytic acid phosphorus, and the amounts of these in the two flours are given in Table 4.

TABLE 4. The calcium, magnesium, potassium and phosphorus in 69 and 92% flours

Mineral element	69% flour mg./100 g.	92% flour mg./100 g.
Calcium	18.5	34.8
Magnesium	38.0	127
Potassium	147	376
Phosphorus (total)	83	287
Phosphorus (as phytic acid)	56	214

In baking the usual type of bread, water, salt and yeast are added to the flour. The quantity of yeast is so small that it may be neglected for the moment. The moisture in a brown loaf, however, amounts to about 43% and in a white loaf to 34%. Bread, therefore, has correspondingly lower concentrations of nitrogen, magnesium, phosphorus, etc., in it than the flour from which it was baked. It has, however, more sodium chloride, and it often has more calcium in it, due to the introduction of this element in the water or as an impurity in the commercial salt. Bread, moreover, contains much less phytic acid phosphorus than can be explained by the changes in moisture. The reduction is due to the enzymic destruction of phytic acid when the bread is set to rise, and does not take place to any extent in the baking of 'soda' bread. In this process the dough is not set to rise at a temperature conducive to enzyme action, but is placed in a hot oven as soon as it is made. The pH, moreover, of the reacting mixture is less favourable for the action of phytase [Widdowson, 1941].

Flours of high extraction have laxative properties. This effect, which is generally attributed to the fibre [McCance & Lawrence, 1929], has been demonstrated repeatedly [Williams, 1927-8; Rose, MacLeod, Vahlteich, Funnell & Newton, 1932; Cowgill & Anderson, 1932], and was discussed by McCance & Widdowson [1940]. In the present experiments the change from 69 to 92% flour doubled the wet and dry weights of the faeces. These bulky stools were not analysed for nitrogen, fat and cellulose residues, but they may be assumed to have contained more

of them all than those passed on the white bread diets [McCance & Widdowson, 1940].

It was thought that some or all of these physiological and chemical changes might influence the bacterial flora in a uniform manner, and that this in turn might have an important effect upon the absorption of some of the mineral elements (possibly by altering the intestinal pH). Accordingly, samples of four of the subjects' faeces were taken for bacterial examination at the end of two white and two brown bread experiments. One pair of these experiments lasted 3 weeks and the other 4 weeks. The examination was very kindly carried out by Dr Crowley. Direct smears were made, and anaerobic cultures of standardized faecal dilutions were set up in various ways, and the colonies counted after 5 days' incubation. The results are given in Table 5, and Dr Crowley considers that the figures show that neither

TABLE 5. Faecal bacteria on white and brown bread diets

Subject	N. K.		R. M.		B. A.		E. W.	
Bread	White	Brown	White	Brown	White	Brown	White	Brown
% total flora present as:								
Coliform bacilli	6	2	9	14	4	6	2	3
Faecal streptococci	3	5	11	9	0	5	7	13
Lactobacilli	63	63	57	69	85	77	70	63
'Bacteroides'	27	27	19	8	10	12	12	21
Other organisms	1	2	4	0	1	0	9	0

diet produced any gross alteration in the relative frequency of the organisms, and it is improbable, therefore, that bacterial variation lay behind any of the definite chemical changes now to be described.

*The absorption and excretion of minerals from white and brown bread dietaries*

*The absorption of calcium.* Table 6 shows the absorption of calcium by all the subjects from white and brown bread dietaries at two different levels of intake. Here, and throughout this paper, the amount of any mineral absorbed has been taken to be the amount in the food minus the amount in the faeces. It will be noted that (a) the intakes tended to be a little higher on brown than on white bread, (b) the absorptions were always lower. Some of the subjects, E. B. for example, absorbed calcium freely, others, such as N. K. and R. M., only with difficulty, but the same change in experimental conditions affected them all in the same way. Hence it may be concluded with confidence that there is something about flour of 92% extraction which has a deleterious effect upon the absorption

TABLE 6. The absorption of calcium from white and brown bread diets

Period of observation ...	Low intakes				High intakes			
	69% flour		92% flour		69% flour		92% flour	
	21 days		21 days		28 days		28 days	
	July		August		Oct., Nov.		Sept., Oct.	
Time of year ...	Ca intake	Absorption	Ca intake	Absorption	Ca intake	Absorption	Ca intake	Absorption
Subject	mg./day	mg./day	mg./day	mg./day	mg./day	mg./day	mg./day	mg./day
E. B.	500	307	530	169	1030	403	1330	317
N. K.	482	130	580	27	1300	279	1470	185
R. M.	720	181	676	20	1390	198	1490	118
P. S.	—	—	—	—	1330	390	1365	253
C. B.	557	178	630	64	—	—	—	—
B. A.	380	121	495	46	1030	178	1030	107
A. M.	416	127	516	74	1150	273	1190	219
E. W.	450	118	550	50	1075	206	1110	114
R. W.	—	—	—	—	885	128	1000	85
K. B.	457	133	520	70	—	—	—	—

of calcium from the human intestine. These experimental findings recall those of Burton [1929-30], who carried out metabolic studies on six children and two adults. Her object was to compare the effects of oatmeal and white wheat flour on the absorption and excretion of calcium, and her work was partly inspired by Mellanby's. Unfortunately, her experimental periods were exceedingly short, the diets did not contain very much of the cereal, and the intakes were not similar enough in the periods to be compared. Nevertheless, her results showed that oatmeal depressed the absorption and retention of calcium, and this was no doubt due to the same phenomenon with which Mellanby had been dealing and which underlies the present results.

*The magnesium balances.* Table 7 shows the magnesium balances of all the subjects on the two kinds of bread. The long periods of observation were due to the fact that changes in the calcium intakes could not be demonstrated to interfere with the magnesium metabolism, so that more experiments could be drawn upon for some of the magnesium data than for any one aspect of the calcium investigation. As may be seen, all the subjects reacted in the same way. They were 'in balance' on the white bread and remained so on the brown. Indeed, in spite of the greatly increased magnesium intake owing to the large quantities of this element in 92% flour, there was little absolute change in the amount absorbed or in the amount excreted in the urine. Both, however, formed a much smaller percentage of the intake when the basis of the diet was 92% wheatmeal. Before passing on from this table, there is one point which may be mentioned. It will be recalled that E. B. was the subject who

TABLE 7. The absorption and excretion of magnesium on white and brown bread diets

Subject	Bread	Period of observation days	Mg intake mg./day	Absorption mg./day	Absorption as % intake	Balance mg./day
E. B.	White	49	317	139	44	+ 3
	Brown	63	729	154	21	+ 2
N. K.	White	56	346	203	59	+ 9
	Brown	98	862	244	28	+ 8
R. M.	White	63	426	180	42	+ 2
	Brown	98	790	193	24	- 6
P. S.	White	42	378	172	46	+13
	Brown	63	719	158	22	- 3
C. B.	White	21	394	157	40	+14
	Brown	21	842	218	26	+42
B. A.	White	56	266	146	55	+12
	Brown	84	583	146	25	- 2
A. M.	White	49	271	131	48	+ 5
	Brown	84	660	174	26	+ 9
E. W.	White	63	318	128	40	+10
	Brown	105	650	145	22	+ 8
R. W.	White	28	213	89	42	-11
	Brown	63	510	115	23	- 6
K. B.	White	21	283	114	40	+16
	Brown	21	633	158	25	+ 2

absorbed calcium most readily, and that N. K. was the reverse. Table 7 shows that N. K. consistently absorbed magnesium better than E. B. This demonstrates very well the individual differences which may be found in mineral metabolism, and for which at present there seems to be no explanation.

The effect obtained when the magnesium intake was increased by adding magnesium carbonate to white bread is shown in Table 8. This

TABLE 8. The effect of adding magnesium carbonate to white bread

Subject	White bread			White bread + MgCO <sub>3</sub>			Brown bread		
	Mg intake mg./day	Absorption mg./day	Urine mg./day	Mg intake mg./day	Absorption mg./day	Urine mg./day	Mg intake mg./day	Absorption mg./day	Urine mg./day
E. B.	310	127	131	590	239	176	568	127	133
B. A.	226	123	116	492	150	187	499	124	146
P. S.	391	169	158	678	237	210	670	171	149
R. W.	199	76	113	450	143	135	459	120	114

The periods of observation on which these results are based varied in length from 14 to 35 days.

gives the results of a small 14-day experiment on four of the subjects. It will be seen—and the results confirm those of Tibbetts & Aub [1937]—that in each subject the addition brought about an increased absorption and a corresponding increase in the urinary excretion. The percentage

absorptions are not shown in the table, but E. B.'s did not alter, the others' fell, but not much. Table 8 also shows the absorptions and urinary excretions of these four subjects on brown bread diets at magnesium intakes selected so that they were comparable with those achieved when the white bread was fortified with magnesium carbonate. The absorptions were lower, and in three subjects they were almost the same as they had been on the unfortified white bread.

These results show that the magnesium in brown bread is not so well absorbed as inorganic magnesium added to white bread and probably less well than the magnesium in white bread itself. They do not, however, demonstrate as conclusively as the previous results did for calcium that there is something in the 92% flour which inhibits the absorption of free magnesium ions. This is so because the 92% flour itself introduced the extra magnesium into the diets, and it would be legitimate, therefore, to argue that all the magnesium therein was so encased by cellulose that it never went into solution in the stomach or gut, and so was never exposed to the chance of absorption. There was not the same ambiguity in the calcium results, for little of the calcium in the diet came from the breads, whether they were made from wheatmeal or from white flour. Further discussion will be deferred till more evidence has been brought forward.

*The potassium balances.* Table 9 shows some of the potassium balances on white and on brown bread diets. The subjects were essentially 'in balance' throughout, but it will be seen that the change from white to

TABLE 9. The potassium balances on white and brown bread

Subject	Bread	K intake mg./day	Excretion			Balance mg./day
			Urine mg./day	Faeces mg./day	Faeces % of intake	
N. K.	White	3820	3030	728	19	+272
	Brown	6230	4760	1440	23	- 30
C. B.	White	4640	3500	636	14	+504
	Brown	6430	4070	1790	28	+670
K. B.	White	3160	2830	585	18	-255
	Brown	4340	3000	1250	29	+ 90
E. W.	White	3980	3240	443	11	+297
	Brown	5350	4500	950	18	-100

Periods of observation: N. K. and E. W., 14 days; C. B. and K. B., 21 days.

brown bread raised the intakes. As in the case of magnesium, this was due to the fact that the 92% flour had much more potassium in it than the white flour. More potassium was absorbed, and in consequence more was excreted in the urine. The faecal excretions were also increased both

absolutely and as a percentage of the intakes. These findings corroborate the preliminary data of McCance & Widdowson [1940], and recall the magnesium results which have just been discussed. Similar reasoning may be applied, but since it is improbable that wheatmeal contains any substance which could precipitate potassium ions from aqueous solution, in the same way that phytic acid precipitates calcium and magnesium, the increased amounts in the faeces may be attributed with more confidence to the indigestible nature of the branny particles, or to their laxative action. It is interesting in this connexion to mention that lithium in natural diets, and particularly these brown bread diets, was very poorly absorbed, whereas added lithium salts were quantitatively absorbed and excreted [Kent & McCance, 1941a].

*The intakes and absorptions of phosphorus.* Table 10 shows the intakes, absorptions, and the percentage absorptions of phosphorus from

TABLE 10. The absorption of phosphorus from white and brown bread dietaries

Subject	White bread (periods of observation 21 days)			Brown bread (periods of observation 21 days)		
	P intake mg./day	Absorption mg./day	Absorption % of intake	P intake mg./day	Absorption mg./day	Absorption % of intake
E. B.	1230	900	73	1970	1060	54
N. K.	1270	978	77	2400	1310	55
R. M.	1530	897	59	2320	1030	44
C. B.	1480	1020	69	2340	1250	53
B. A.	995	705	71	1850	920	50
A. M.	880	620	71	1660	880	53
E. W.	1120	744	67	2120	1170	55
K. B.	1140	760	67	1800	998	55

TABLE 11. The absorption of phosphorus from diets with and without added inorganic phosphate

Subject	White bread				Brown bread			
	Low P intake 7-day periods		High P intake 14-day periods		Low P intake 14-day periods		High P intake 7-day periods	
	Intake mg./day	Absorption % intake	Intake mg./day	Absorption % intake	Intake mg./day	Absorption % intake	Intake mg./day	Absorption % intake
E. B.	1130	69	1810	66	2360	50	2830	52
N. K.	1310	70	1730	72	2620	48	2910	56
R. M.	1400	55	1890	59	2490	41	2770	48
P. S.	1370	61	1910	66	2340	47	2860	56
B. A.	1080	63	1510	62	1760	42	2100	44
A. M.	1000	66	1410	66	1750	47	2040	55
E. W.	1270	60	1650	60	1980	46	2320	49
R. W.	990	57	1230	63	1680	46	2020	48

white and brown bread diets, and Table 11 the intakes and percentage absorptions from similar diets to which calcium carbonate or calcium monohydrogen phosphate had been added. The balances have not been

given for they have shown no alterations, any increase or decrease in the amounts absorbed being followed by a corresponding change in the amount excreted in the urine.

It will be noted that all the subjects had larger absorptions of phosphorus on the brown bread diets than they had on the white, but that these represented a smaller percentage of the intakes. When inorganic phosphate was added to the diets, the subjects absorbed this as freely as the phosphorus in white bread and more so than the phosphorus in brown. This is shown in Table II by the fact that the percentage absorptions did not change when the additions were made to white bread, but all rose when the additions were made to brown. Evidently, therefore, much of the phosphorus in brown bread is less readily absorbed than added inorganic phosphorus, or than the phosphorus in white bread. These observations should probably be explained as follows: The phytic acid phosphorus in brown bread is partially broken down in the gut to inorganic phosphorus, which is then absorbed. This explains most of the increased absorption, but some is no doubt due to the fact that brown bread contains not only much more phytic acid but also a little more inorganic phosphorus than does white. The smaller percentage absorption is due to two causes, of which the relative unavailability of phytic acid phosphorus is the main one, and the laxative effect of brown bread a subsidiary one.

#### *The role of phytic acid*

*The addition of sodium phytate to white bread.* In the experiment now to be discussed eight people have eaten diets similar to those already described, but 40–50% of their calories were derived from white bread to which sodium phytate had been added. Thus it was possible to investigate the effect of phytic acid on the absorption of calcium, magnesium and phosphorus without the complicating laxative action of the brown bread. The sodium phytate was prepared and used as follows: 120 g. of commercial 'Phytin' (Messrs Ciba Ltd.) were dissolved in warm water with the addition of 10 c.c. of concentrated HCl. To this were added 48 g. of sodium oxalate dissolved in 100 c.c. of boiling water. The pH of the mixed solutions was adjusted to 5 by the addition of (about) 20 c.c. of N/10 NaOH. After allowing 2 hr. for the precipitate to settle, this latter was collected on a fluted paper and washed with hot water. The pH of the filtrate was next adjusted to 7 by the further addition of (about) 19 c.c. of N/10 NaOH. If a cloud formed, this was filtered off; if not, this step was omitted, and the solution made up to a total volume of 2000 c.c. After making up to volume, the solution was tested with



ammonium oxalate to make sure that there was still a faint trace of calcium in the preparation, and hence that no oxalate was present. The yield of phytic acid phosphorus was almost quantitative and the solution contained 1.1 % phytic acid phosphorus.

This solution of sodium phytate was used for preparing the dough when the bread was being baked. 50 c.c. (0.55 g. of phytic acid phosphorus) were incorporated into each 1 lb. loaf, and the bread was baked with sodium bicarbonate and potassium hydrogen tartrate. This was done for reasons of economy because baking with yeast destroys part of the phytic acid [Widdowson, 1941]. The bread so prepared contained about 130 mg./100 g. of phytic acid phosphorus. This was rather more than the brown bread previously used, for which the corresponding figure was about 100 mg. The sodium phytate solution was also used in some of the puddings, cakes and pastries at the rate of 100 c.c. (1.1 g. of phytic acid phosphorus) to 1-1½ lb. of flour. Had the puddings and pastries not been so treated, the daily intake of phytic acid would have been roughly the same as it had been on the brown bread. As it was, it was somewhat higher.

When the decision was taken to bake the bread with baking powder instead of yeast it was not appreciated that citrates and tartrates had been shown to prevent the high-calcium low-phosphorus type of rickets in rats [Shohl, 1937; Hamilton & Dewar, 1937; Hathaway & Meyer, 1939; Day, 1940]. If their action were due to the unionized complexes which they may form with calcium salts, then presumably such acids might influence unfavourably the absorption of calcium from low-calcium high-phosphorus diets. There is, however, no evidence that they do any such thing [Bauman & Howard, 1912; Hess & Matzner, 1924; Chaney & Blunt, 1925; Weissenberg, 1924; Siwe, 1938; Shohl & Butler, 1939]. In fact, citrates seem to promote calcium absorption in man, and therefore they would probably minimize rather than exaggerate the bad effects of phytic acid about to be described. As a final precaution, however, the bread was baked for one week with an acid phosphate instead of tartrate, and no differences could be detected.

*The effect of sodium phytate on the absorption of calcium and other bases.* Table 12 shows how the absorptions and urinary excretions of calcium were changed when sodium phytate was added to white bread. All the subjects reacted in the same way. Their urinary calcium fell, their absorptions went down so much that a number of the subjects (notably N. K., R. M. and E. W.) actually showed quite large negative quantities in this column. This means that there was more calcium in their faeces

TABLE 12. The effect of sodium phytate on the absorption and excretion of calcium

Subject	White bread				White bread + sodium phytate			
	Period of observation days	Ca intake mg./day	Absorption mg./day	Urine mg./day	Period of observation days	Ca intake mg./day	Absorption mg./day	Urine mg./day
E. B.	21	500	307	254	21	453	107	155
N. K.	14	540	141	214	14	540	- 111	128
R. M.	14	645	36	97	21	630	- 105	46
P. S.	14	578	216	216	21	597	- 9	168
B. A.	21	510	63	109	21	436	- 11	57
R. W.	14	525	157	139	21	432	13	64
A. M.	21	416	127	122	21	518	6	86
E. W.	14	440	41	84	21	476	- 46	46

than there had been in their food, and it is explained by the fact that they must have failed to reabsorb much of the calcium in their intestinal secretions. It is obvious that if a person's faeces contain more calcium than his food he must be in negative balance. As a matter of fact all these subjects were in negative balance when their bread contained sodium phytate, for their urinary output was always greater than their alimentary absorption. The whole question of calcium balances will, however, be deferred for the present and discussed fully when calcium requirements are being considered.

The addition of sodium phytate to white bread, therefore, reproduced in an exaggerated degree the effect of brown bread on calcium metabolism. In these experiments, however, there were none of the laxative effects of brown bread. In fact, none were expected, for, in spite of some earlier confusion [Jordan, Hart & Patten, 1906; Mendel & Underhill, 1906; Williams, 1927-8], it now seems to be established that the laxative action of whole cereals is due to the indigestible nature of the branny particles and not to phytic acid [Cowgill & Anderson, 1932]. Hence the whole of the changes in the metabolism of calcium shown in Table 12 may be attributed to the chemical action of the added sodium phytate.

Table 13 shows how the metabolism of magnesium was altered by sodium phytate. As with calcium, the absorptions and the urinary excretions fell. Obviously sodium phytate was affecting magnesium metabolism in very much the same ways as calcium metabolism. (This additional evidence, if such were needed, indicates that the calcium effects were not due to the tartrate used in the baking powder.) The balances, however, were not made negative, and there was never any suggestion that the amount of magnesium in the faeces might exceed that in the food. Again, sodium phytate had reproduced the effects of brown bread.

TABLE 13. The effect of sodium phytate on the absorption and excretion of magnesium

Subject	White bread				White bread + sodium phytate			
	Mg intake mg./day	Absorption mg./day	Urine mg./day	Balance mg./day	Mg intake mg./day	Absorption mg./day	Urine mg./day	Balance mg./day
E. B.	310	127	132	- 5	308	86	90	- 4
N. K.	365	218	229	- 11	439	129	125	+ 4
R. M.	447	214	182	+ 32	490	140	137	+ 3
P. S.	392	169	156	+ 13	419	92	100	- 8
B. A.	304	146	162	- 16	296	116	97	+ 19
A. M.	333	153	156	+ 3	347	103	99	+ 4
E. W.	324	138	125	+ 13	350	83	82	+ 1
R. W.	229	103	103	± 0	251	83	72	+ 11

The periods of observation on white bread were in general longer than those in Table 12, those on white bread + sodium phytate were the same length.

In one way, however, sodium phytate did not mimic the effects of brown bread. Table 14 shows that the phytate had no inhibitory effect on the absorption of potassium. None was expected, but the negative result makes a satisfactory control. Moreover, it enhances the probability (a) that sodium phytate inhibits the absorption of calcium and

TABLE 14. The effect of sodium phytate on the absorption of potassium

Subject	White bread		White bread + sodium phytate	
	K intake mg./day	Potassium in the faeces as % of intake	K intake mg./day	Potassium in the faeces as % of intake
E. B.	3700	16	4010	16
N. K.	3970	19	5010	17
R. M.	5550	23	5490	28
P. S.	4920	13	5380	16
B. A.	3080	15	3360	12
A. M.	3270	13	3740	18
E. W.	4160	13	4470	14
R. W.	2530	19	2460	17

The periods of observation varied from 14 to 35 days.

magnesium by a specific chemical action, and (b) that the inhibitory effect of brown bread on the absorption of potassium is due to some less specific effect—probably to the indigestible nature of the bran.

*The metabolism of phytic acid phosphorus.* Table 15 shows the intakes, absorptions and percentage absorptions of phosphorus from white bread and from white bread to which sodium phytate had been added. The subjects were in balance on both diets, so that the urinary excretions closely followed the absorptions, and have been omitted from the table. If it be assumed that the increased intake of phosphorus shown by all the subjects was due to the added sodium phytate, which is approximately true, it is possible to make an estimate of its absorption, and this has been

TABLE 15. The absorption of phytic acid phosphorus

Subject	White bread			White bread + sodium phytate			% absorption of P added as sodium phytate
	Total P intake mg./day	Absorption mg./day	Absorption % of intake	Total P intake mg./day	Absorption mg./day	Absorption % of intake	
E. B.	1230	900	73	1840	1150	62	41
N. K.	1270	978	77	2670	1700	64	51
R. M.	1390	870	63	2480	1350	55	44
P. S.	1290	870	67	2260	1300	57	44
B. A.	1000	750	71	1750	1130	65	56
A. M.	995	620	71	1790	1130	63	56
E. W.	1160	765	66	2010	1230	61	55

done in the last column of the table. The figures show quite clearly that the addition of sodium phytate was followed by an increased absorption of phosphorus. Some of the phytic acid phosphorus, therefore, was certainly broken down in the intestine, and became available. A comparison of columns 3, 6 and 7 suggests that the added phytic acid phosphorus was not so readily absorbed as the phosphorus in white bread, and the approximation may be made that 70 % of the phosphorus in the diets was absorbed, and 50 % of the phosphorus introduced as sodium phytate. In general these results uphold the view that the phosphorus in phytates is relatively unavailable [McCance & Widdowson, 1935], but they also show that phytates cannot be disregarded as a source of phosphorus. McCance & Widdowson found that, of the phytic acid phosphorus ingested by three adults, 36–63 % was excreted unchanged in the faeces. The present results provide evidence as to the fate of the remainder. The extent to which phytates are broken down and their phosphorus absorbed probably depends upon many things, but the intestinal flora and the amount of calcium in the diet [Lowe & Steenbock, 1936] are probably the two that matter most.

Putting the calcium, magnesium and phosphorus results together, it is interesting that sodium phytate should be able to prevent the absorption of so much calcium and magnesium, and yet itself be broken down freely enough to allow half of its phosphorus to be absorbed. This suggests that the two metals, and particularly the calcium, can only be absorbed high up in the intestine. Once past the level at which the metals can pass through the wall of the gut, the phytates may be destroyed and the phosphorus absorbed, leaving the metals to be excreted in the faeces.

*Phytate equilibria in vitro.* Harrison & Mellanby [1939] concluded their paper with a thoughtful discussion of the rachitogenic actions of commercial 'Phytin', sodium phytate, and oatmeal. They made the point that for dogs and humans the rachitogenic action of any compound or

foodstuff depended upon the amount of phytic acid and the amount of calcium in it. A low calcium/phytic acid ratio was the real criterion of whether a substance would produce rickets. In their own words 'the degree of active interference with calcification produced by a given cereal will depend upon how much phytic acid and how little calcium it contains'.

Consideration of the present results and the known properties of phytic acid suggested that this was inherently sound reasoning, but that it did not go quite far enough. Phytic acid forms insoluble magnesium, zinc, iron and copper salts as well as an insoluble calcium salt, and insoluble mixed salts are also well known. The ferric salt is highly insoluble, even in  $N/6$  HCl, and is used for the isolation and determination of phytic acid. The calcium salt is soluble at pH 2.8, but precipitation begins when the pH rises to 3.0. The magnesium salt is soluble up to about pH 5. These facts raise two questions. First, to what extent can magnesium and other metals in a natural diet saturate the phytic acid and so prevent it exerting its rachitogenic effect? Secondly, why does the presence of phytic acid in a diet not produce severe anaemia, or even a zinc deficiency? Experiments have been undertaken *in vitro* to try to answer these questions.

In practice there is not enough iron or zinc in a diet to influence the precipitation of calcium by phytic acid. There is, however, enough magnesium, for some of the foods which contain phytic acid also contain really large concentrations of magnesium, whole wheat being an excellent example. Further, the magnesium results which have just been given show that the metabolism of this element is affected by phytic acid.

Even if experiments *in vitro* are restricted to calcium, magnesium and phytic acid, matters are extremely complicated, for the number of variables is so great. The relative concentrations of calcium, magnesium, total base and phytic acid, and the pH, all affect the amount and the composition of the phytates which are precipitated. These variables have been investigated, although naturally not in every particular, but the presentation of all the results in quantitative tabular form is out of the question. Many of the experiments were carried out at pH 6.5, which may be assumed to be the approximate pH of the intestine. As a matter of fact, the truth of what follows would not be materially altered if the actual pH were 6 or 7. It has been found that at this pH the composition of the mixed phytates varies with the relative concentrations of the bases and the ratio of total base to phytic acid in the solution in which the precipitates are formed. If this solution contained twice as

many equivalents of magnesium as calcium, and if the bases were in excess by three to one, the whole of the phytic acid was precipitated and equal equivalents of calcium and magnesium with it. As the phytic acid in such a solution was reduced, less total base was, of course, precipitated, but the ratio of calcium to magnesium in the precipitate tended to rise. As the phytic acid was increased, the whole of it continued to be precipitated fully saturated with base, so that when equal equivalents of total base and of phytic acid were present in the original solution, for practical purposes the precipitate contained them all, and the calcium/magnesium ratio in the precipitate was 1/2. As the phytic acid in the original solution was further increased, the organic phosphate continued to be precipitated fully saturated with base, but the quantity of the precipitate decreased, so that when the ratio of total base to phytic acid phosphorus in the original solution was 1/3 only 85% of the calcium was precipitated and 58% of the magnesium, and the calcium/magnesium ratio in the precipitate was 1/1.6.

At pH 6.5 no excess of magnesium ions likely to be achieved in practice will prevent small quantities of calcium being precipitated. When 11.65 equivalents of magnesium, 1.12 of calcium and 6 of phytic acid phosphorus were mixed together, 90% of the calcium was precipitated. Yet calcium never completely displaces magnesium from precipitation, for when the equivalents of calcium were raised to 11.65 and the original amounts of magnesium and phosphorus retained, 23% of the magnesium was still precipitated and the calcium/magnesium ratio in the precipitate was 1.8/1. This demonstrates the immobilizing action of phytic acid on small amounts of calcium, but it also shows that calcium will never be completely precipitated in the presence of abundant magnesium ions.

When the pH was reduced below 6.5 in the presence of a total base/phytic acid phosphorus ratio of 3/1, and a magnesium/calcium ratio of 2/1, the whole of the phosphorus was precipitated down to pH 6, and below this progressively less. At the same time the calcium/magnesium ratios in the precipitate tended to rise.

These rather complicated effects all go to show that there are two main clues to the composition of mixed phytates, and indeed to the general physiological behaviour of phytic acid. The first is that the magnesium salt is somewhat more soluble than the calcium salt at pH's between 6 and 7, and that it becomes more soluble still as the pH falls below 6. The second is that mass action among the reactants plays a very large part in determining the composition of the products. These two

general principles are well illustrated by another experiment which has been carried out. Solid magnesium phytate was added to a large excess of calcium chloride at pH 6.5. The suspension was well shaken and left to stand for 24 hr. At the end of this time the precipitate was separated, washed and analysed, and was found, within the limits of experimental error, to consist of pure calcium phytate. Complete replacement had taken place. When, however, the converse experiment was performed, the precipitate analysed after 24 hr. contained 61 equivalents of calcium and 49 equivalents of magnesium per 100 equivalents of phytic acid phosphorus. Replacement of calcium by magnesium had taken place, but it had been incomplete.

One observation has been made in studying the mixed phytates which cannot be explained along these lines. When the ratio of total base to phytic acid phosphorus in the original solution was about 1, the equivalent weights of calcium plus magnesium in the precipitate were equal to those of phosphorus (assuming phytic acid phosphorus to be divalent). This is the expected composition of a saturated phytate. Yet when the bases were present in excess, the equivalent weights of calcium plus magnesium invariably exceeded those of phytic acid phosphorus, and the effect was progressive. When, for example, the original solution contained 23 equivalents of base and 6 of phytic acid phosphorus, the precipitate contained 8 equivalents of base and 6 of phosphorus. It is not easy to picture the way in which the excess of base is combined with the phytic acid. It can hardly be attached to OH groups in the benzene ring of the inositol, for these are occupied by phosphorus.

To make the conditions *in vitro* a little more realistic solutions of calcium, magnesium and sodium phytate were prepared and mixed in the proportions in which these reactants were present in one of E. W.'s brown bread diets and in B. A.'s white bread diet to which sodium phytate had been added. The precipitates which formed at pH 6.5 were analysed, and the results are shown in Table 16. Considering the brown

TABLE 16. The precipitation of phytates from artificial diets

Type of diet		Composition of diet, i.e. of original solution		Composition of precipitate m.oq./day
		mg./day	m. eq./day	
Brown bread	Calcium	505	25.2	17.4
	Magnesium	655	54.6	18.8
	Phytic acid P	480	31.0	30.4
White bread +sodium phytate	Calcium	478	23.9	23.2
	Magnesium	350	29.1	26.2
	Phytic acid P	766	49.5	48.8

bread diet first, it will be seen that in it there was an excess of base of nearly 3 to 1 and an excess of magnesium over calcium of slightly more than 2 to 1. (In 100 g. of brown bread itself there are 6.45 m. eq. of phytic acid phosphorus, 6.9 m. eq. of magnesium and 1.8 m. eq. of calcium, so that the bases slightly exceed the acid, and magnesium is present in great excess.) 99% of the phytic acid phosphorus was precipitated, and slightly more magnesium than calcium. The excess of base over acid in the precipitate will also be noted. This reproduction of actual conditions demonstrates rather well that, owing to the greater insolubility of the calcium salt, even a large excess of magnesium will not prevent most of the calcium being precipitated, so that on such diets phytic acid must be expected to depress the absorption of free calcium ions to a greater extent than it depresses the absorption of free magnesium ions. The practical difficulty of testing this lies in the fact that all the magnesium ions in 92% flour may not be 'free'. Turning to the other diet, it will be seen that there was a bare excess of base over acid in the original solution, and only a slight excess of magnesium over calcium. There was, moreover, more phytic acid than in the brown bread diet. Almost the whole of this last was precipitated, and 97% of the calcium. Owing to the excess of base being so small, there was also room on the precipitate for 90% of the magnesium, and the whole of this came down, so that the precipitate contained more equivalents of magnesium than calcium. This excess, moreover, slight though it was, was greater than it had been on the brown bread precipitate, in spite of the fact that the latter had been formed in the presence of a much greater magnesium preponderance. It is in conditions such as these that phytic acid might be expected to exert its maximum effect in depressing the absorption of free magnesium and calcium ions, and the metabolism experiments bore this out.

The second question which has been raised, namely, what is the effect of phytic acid on the metabolism of iron and divalent metals other than calcium and magnesium, will be discussed on another occasion.

#### *Cereal diets and calcium requirements*

If the calcium in the faeces is subtracted from the calcium in the food, the remainder represents the amount 'absorbed and available for the body's use'. If this quantity is as great as the amount of calcium in the urine, the person is in 'balance', and his immediate needs for calcium are being met. The smallest absorption, therefore, which will maintain balance may be defined as the true or internal requirement. The



magnitude of this figure depends only upon an individual's intermediary calcium metabolism, and is independent of the food. The smallest amount of calcium which has to be taken by mouth to maintain balance, i.e. to provide the true or internal requirement, is often spoken of as the minimum requirement. Its value depends partly upon the individual's internal requirement and partly upon his ability to absorb calcium, but fully as much upon the nature of his food. He will absorb calcium badly if his diet contains much bread made from 92% flour. All dietary requirements for calcium, therefore, must be defined in terms of the staple cereal. This has not been appreciated, and has never up till now been done. Table 17, however, shows how essential it is to do so. In

TABLE 17. Calcium balances on white and brown bread diets

Subject	White bread		Brown bread	
	Ca intake mg./day	Balance mg./day	Ca intake mg./day	Balance mg./day
E. B.	500	+ 54	530	- 10
P. S.	642	+ 1	550	- 97
N. K.	482	- 118	560	- 190
R. M.	720	- 43	676	- 150
B. A.	380	- 9	495	- 74
C. B.	557	- 27	630	- 111
K. B.	457	- 23	520	- 54
A. M.	416	+ 4	516	- 26
E. W.	450	- 4	550	- 73

Periods of observation 21 days.

considering these results it is best to disregard small positive and negative balances, for they may have been technical or fortuitous. The difficulty is to know what limits to put upon this range, but assuming that balances between + and -25 mg./day are not significantly positive or negative, then Table 17 shows that seven subjects were in satisfactory balance on the white bread diets. There was, therefore, enough calcium in this food. Only R. M. and N. K. were well outside the range of  $\pm 25$  mg./day, and these two clearly needed more calcium. In contrast, Table 17 shows that only one person remained in balance when 92% flour was substituted for the 69%. The intakes were higher in nearly every case, but they did not satisfy the subjects' requirements. E. B. was able to obtain enough calcium from these diets, and he must be regarded as exceptionally good at this, for his percentage absorptions were always very high as judged by others in this investigation, and by data culled from the literature.

To change a nation's dietary from white to brown bread, and at the same time to reduce their milk and cheese supply, would probably mean

that nine adults out of ten would begin to lose calcium. No data are available for children, but it may be predicted that if such a change were made their storage of calcium would deteriorate. Rickets might increase in young children and growth become slower at all ages.

### *Vitamin D and calcium absorption*

Mellanby [1925, 1934] has always stressed the great value of vitamin D in correcting the rachitogenic action of oatmeal diets in puppies, and in improving the calcium metabolism of children. Others have shared this view, and when the bad effects of 92% flour on the absorption and balance of calcium were first observed it was thought that vitamin D would probably put them right. Accordingly, the effect of vitamin D was investigated. Throughout this 3-week experiment and for 7 days before it, 2000 I.U. of vitamin D as calciferol were given daily to each subject. The drug, which was given to us by Messrs Glaxo Ltd., was made up in arachis oil and eaten at lunch-time on a crust of bread. 92% flour was used as the basal cereal because it was thought that on it vitamin D would have a chance to show what it could do. Special 14-day control periods were necessary for some of the subjects, whose calcium metabolism had changed since their original 3 weeks on brown bread 6 months before. The results are shown in Table 18, and will

TABLE 18 The effect of vitamin D on the calcium absorptions and balances

Subject	Brown bread				Brown bread + 2000 I.U. calciferol/day			
	Ca intake mg/day	Absorption mg/day	Urine mg/day	Balance mg/day	Ca intake mg/day	Absorption mg/day	Urine mg/day	Balance mg/day
E. B.	530	169	179	- 10	476	131	179	- 47
P. S.	550	23	120	- 97	540	57	111	- 54
N. K.	662	4	131	- 127	610	31	127	- 96
R. M.	656	- 97	27	- 124	701	- 53	29	- 82
B. A.	499	16	74	- 59	501	7	46	- 39
A. M.	516	74	100	- 26	607	69	99	- 30
R. W.	472	- 33	86	- 119	483	- 73	64	- 137
E. W.	518	- 33	41	- 74	522	41	47	- 6

Periods of observations on brown bread alone 21 or 14 days and on vitamin D 21 days

disappoint those who regard vitamin D as a universal restorative. Four of the subjects—E. B., B. A., A. M. and R. W.—absorbed less calcium under the influence of vitamin D, and E. B., A. M. and R. W. had worse balances. The others absorbed more and had rather less negative balances, but only E. W. showed any real betterment from D, and even her increased absorption was not enough to produce a positive balance. A study of the literature then showed that this was really the result to have been expected, taking into account the dosage of D and the age and

health of the subjects. Our knowledge about the action of the vitamin in adults may be summarized as follows. Whatever the other effects of vitamin D there is no doubt that even small doses greatly improve the calcium absorptions of adults with osteomalacia. Chu, Liu, Yu, Hsu, Cheng & Chao [1940] found that even one egg a day made a difference to the calcium absorptions, and 500 I.U./day produced a large effect, which lasted for some weeks after the drug was stopped. Chu, Yu, Chang & Liu [1939] showed that sunlight and irradiation had good effects in quite moderate dosage, and there is an extensive literature on the beneficial effects of larger doses [Hannon, Liu, Chu, Wang, Chen & Chou, 1934; Liu, 1940; Liu, Hannon, Chu, Chen, Chou & Wang, 1935; Liu, Hannon, Chou, Chen, Chu & Wang, 1935; Liu, Su, Wang & Chang, 1937; Liu, Chu, Su, Yu & Cheng, 1940; Liu, Chu, Hsu, Chao & Chen, 1941]. In some of this work the periods have been too short and the subjects have not always been given enough time to come into equilibrium after a change of diet. (See particularly Liu, Hannon, Chu *et al.* [1935].) Nevertheless, the osteomalacia results as a whole are indisputable. Bauer & Marble [1932] showed that 5 mg. of irradiated ergosterol per day produced a very slight improvement in the calcium absorption of a patient with osteoporosis; 8 and 20 mg./day produced very definite effects, but these were enormous doses. Adams, Boothby & Snell [1935-6] claimed to get an improved absorption on giving 30 minims of viosterol per day to a patient with osteoporosis, but their results are not convincing. Most people have given large and variable doses of calcium as well as vitamin D [Meulengracht & Meyer, 1937], and their results are of therapeutic interest but of no value to the present discussion. On the other hand, all those workers who have administered physiological doses of vitamin D to normal adults have found that it did not alter the calcium absorptions, urinary excretions, or balances. Hunscher, Donelson, Erickson & Macy [1934] used 15 g. of cod-liver oil per day as the source of vitamin. Kroetz [1927] used quartz lamp irradiation, but he had only one subject (himself). Hart, Tourtellotte & Heyl [1928] are often quoted as having found that neither cod-liver oil nor irradiation affected the calcium balance of adults, but they had only one subject, and although the periods were long they unfortunately irradiated the subject in the first period and used the second as their control. The prolonged action of vitamin D makes their experiment of little value. Henderson & Kelly [1929-30*a, b*] and Kelly & Henderson [1929-30] used African natives as subjects, and gave each of them 15 c.c. of cod-liver oil per day. The experiments were well conceived, but the

metabolic periods were too short. Nevertheless, their results seem definite enough. Buttner's [1939] experimental periods were long. The excretion of calcium by the kidney was quite unaffected by a dose of cod-liver oil containing 100 i.u. per day, but no other data were given. Johnson [1937] gave 3 c.c. of viosterol, which apparently contained 750 units of D, to patients with ileostomies. Some changes in the ileal discharges were noted, but no improvement in the absorption of calcium. Bauer, Marble & Clafin [1932] found that 5-20 mg. of irradiated ergosterol produced only slight and indefinite effects on the calcium metabolism of normal persons. They stated in their paper that 0.0001-0.00025 mg. of their preparation of ergosterol protected rats against rickets, so that they seem to have been giving 50,000-200,000 i.u. of the vitamin per day, doses which were quite outside physiological possibilities. When they raised the dose of irradiated ergosterol still further to 30 mg./day they noted that less calcium was excreted in the faeces and more in the urine, but that the balances remained unchanged. This dose was so colossal that the results have really no bearing on the present issue.

Although some have argued otherwise, our subjects must be regarded as having been normal. Even R. M. and N. K., who had been in negative calcium balance for some months, only lost 10 or 12 g. of calcium in the whole series of experiments, and these amounts could hardly be considered to have depleted their body stores to a serious extent.

The present results therefore confirm those of Kroetz [1927], Henderson & Kelly [1929-30*a, b*], Hunscher *et al.* [1934], and Buttner [1939]. Vitamin D, given in physiological doses, does not improve the calcium absorptions of normal adults. The results, of course, do not suggest that vitamin D has no action upon adults, or that adults do not require vitamin D. The vitamin's action on the absorption and excretion of calcium is the only one which has been, and possibly ever can be, studied directly in man. Many have thought this the only, or certainly the primary action of the vitamin [Harris, 1934; Nicolaysen, 1937*a, b, c*], but some have always believed that the important action of D was on the internal metabolism of bone [Light, Miller & Frey, 1929; Brown & Shohl, 1930; Taylor & Weld, 1932; Schneider & Steenbock, 1939]. This opinion has been reiterated by Cohn & Greenberg [1939] and Morgareidge & Manly [1939], who have been working with radioactive phosphorus in rats. The present results have no bearing on this aspect of vitamin D's activity.

*The fortification of bread with calcium*

In an experimental study of rationing which was carried out by McCance & Widdowson [1940] in the first 6 months of the war, small amounts of precipitated chalk were added to the bread. This was done to prevent the intakes of calcium being curtailed by the experimental restrictions of milk, cheese and eggs. This fortification of the bread did not impair its palatability, but it was not possible to find out its effect upon calcium absorption, because metabolism experiments did not form part of that investigation. When, therefore, brown bread was found to depress the absorption of calcium, it was natural to try whether fortifying it with calcium carbonate would overcome this obvious defect. The situation became more complicated when the Government proposed to fortify white flour with a calcium salt, for the suggestion aroused noisy, if not perhaps very scientific, opposition. Some workers, with experience of calcium and phosphorus metabolism in rats, suggested that the proposal to use the carbonate was an unwise one. They argued that such an addition would merely defeat its own ends by raising the intake of calcium, but not of phosphorus, and by so doing make the calcium/phosphorus ratio in the diet less favourable to calcium absorption and to bone formation than before. If, therefore, the object of the Government's move was to improve the absorption and metabolism of calcium and phosphorus, then calcium mono-hydrogen phosphate was the salt to use. Accordingly, in September 1940, the programme of these experiments was extended to include a fortified white bread, and to compare the merits of the carbonate and phosphate.

There seemed to be three main questions to be answered:

- (1) What were the relative advantages of calcium carbonate and calcium phosphate?
- (2) Did the absorptions of calcium satisfy the requirements of the subjects when the bread was fortified?
- (3) Should calcium be added to the nation's bread, and if so, how much?

*Carbonate or phosphate?* Table 19 shows the answer which was obtained to this question. The calcium salts were added to the flour so that 100 g. of bread contained 0.1 g. of added calcium. The results have been condensed by separating the four men from the four women and averaging each set of data. Since brown bread is known to inhibit the absorption of calcium it was necessary to see that the proportions of calcium and of flour in the diets did not vary enough to vitiate the

TABLE 19. The absorption of calcium from flours fortified with calcium carbonate and calcium monohydrogen phosphate

Type of flour and designation of subjects	Carbonate				Phosphate			
	Ca/flour ratio	Average intake of calcium mg./day	Average absorption mg./day	Average absorption % of intake	Ca/flour ratio	Average intake of calcium mg./day	Average absorption mg./day	Average absorption % of intake
69% flour:								
4 men	2.76	1250	340	26	2.82	1270	294	23
4 women	2.91	1040	189	18	2.87	1030	202	20
92% flour:								
4 men	2.93	1330	201	15	3.25	1430	202	14
4 women	3.26	1015	110	11	3.49	1110	142	13

*Note.* In calculating the calcium/flour ratio, the calcium was reckoned in milligrams and the flour in grams. The periods of observation varied from 14 to 28 days.

comparisons. The calcium/flour ratios, therefore, as well as the calcium intakes are given in the table. It will be seen that the absorption of calcium was equally good, whether the bread was fortified with the carbonate or the phosphate, so that there was nothing in this respect to choose between them. This being so, there are two good reasons for preferring the carbonate. The first is its abundance, of which no more need be said. The second is its action in the baking and cooking processes. In our experience the mono-hydrogen phosphate does not go so well into the bread. The phosphated white loaf has never been quite so appetizing as its unfortified counterpart, whereas the carbonated loaf has been, if anything, more so. Phosphated brown bread is similar in taste to unfortified bread but its appearance has betrayed it, for even when the salt has been added to the flour as a fine anhydrous powder, white specks have always been observed in the finished article, so that the adulteration has been easily detected by the naked eye. For puddings and pastries made from white flour, there is again a case for the carbonate. Phosphate added to brown flour used for puddings and pastries did not spoil the taste but the white specks were always visible. Carbonate seemed to improve the taste and certainly did not detract from the appearance.

*Calcium absorptions on fortified bread.* Table 20 gives the intakes, absorptions and balances of the subjects on white and brown breads which had been fortified so that 100 g. contained 0.1 g. of added calcium. The flour used for other cooking was also fortified, and to the same extent. The carbonate and phosphate periods have been combined in making up this table since the two salts seemed equally efficacious. If this table is compared with Tables 6 and 17 it will be seen how fortifying the bread altered the intakes, absorptions and balances.

TABLE 20. Calcium intakes, absorptions and balances on diets fortified with calcium salts

Subject	White bread			Brown bread		
	Ca intake mg./day	Absorption mg./day	Balance mg./day	Ca intake mg./day	Absorption mg./day	Balance mg./day
E. B.	1030	403	+ 49	1330	317	+20
N. K.	1300	279	+ 20	1470	185	-46
R. M.	1390	198	+ 14	1490	118	- 2
P. S.	1330	390	+ 14	1365	253	-68
B. A.	1030	178	+ 23	1030	107	-12
A. M.	1150	273	+120	1190	219	+58
E. W.	1075	206	+ 95	1110	114	+27
R. W.	885	128	- 28	1000	85	-48

Periods of observation on white bread 28 days. Periods of observation on brown bread 28 days (E. B., B. A., P. S., R. W.), 49 days (N. K., R. M., A. M., E. W.).

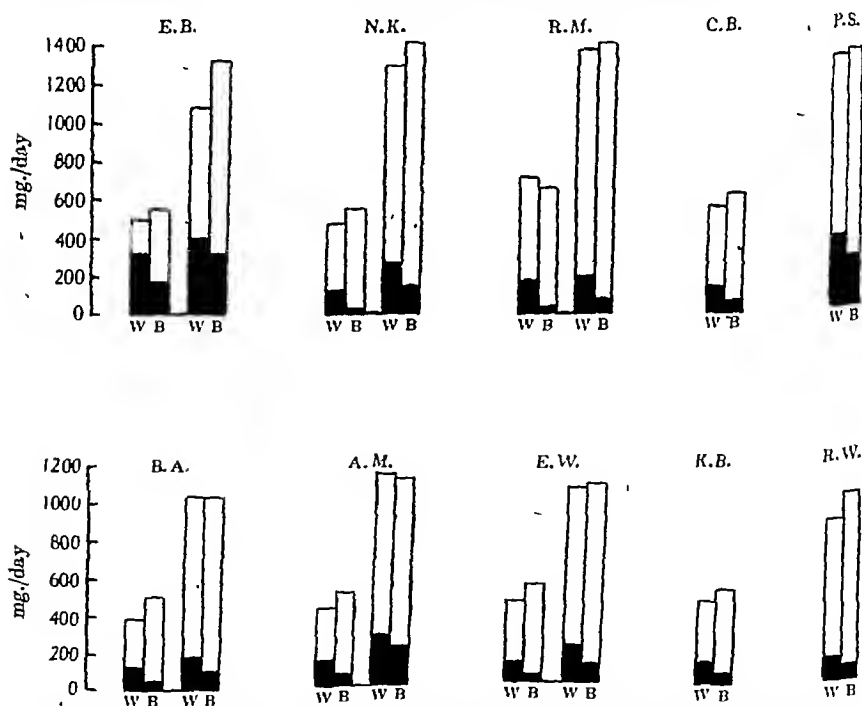


Fig. 1. The absorptions of calcium from unfortified and fortified white and brown bread. W=white bread, B=brown bread. The total heights of the columns represent the intakes, the heights of the solid portions, the absorptions.

Adopting the same convention as before, that persons gaining or losing less than 25 mg. calcium per day should be regarded as being in balance, the fortification of white bread can be said to have maintained all the subjects (even N. K.) in balance or positive balance, with the possible

exception of R. W. Fortifying the brown bread did not quite suffice to keep N. K., P. S. and R. W. in balance. The contrast between Tables 17 and 20 in this respect is striking. Fig. 1 sets out graphically the intakes and absorptions on the white and brown breads, fortified and unfortified. The balances have had to be omitted for the sake of clarity, but the effects of fortification on the absorptions can now be seen at a glance. Two points stand out clearly. The first is that fortification always raised the absorptions and improved the balances if the latter required it. This is so interesting if one recalls that vitamin D failed to do so, and Henderson & Kelly [1929-30] must be given the credit of reaching the same conclusion on the basis of much less satisfactory experimental results. The second is that only a small proportion of the added calcium was absorbed, so that quite a bold addition must be made to effect any real improvement in a person's absorption. There appears, however, to be an interesting difference in this respect between white and brown breads. When white bread was fortified with calcium the percentage of the intake which was absorbed invariably fell. It is impossible to say why this should have been so, but there are several possible explanations, all rather speculative. When, however, brown bread was similarly fortified the percentage absorption rose in all subjects except E. B., who, as already mentioned, absorbed calcium so well from a low intake that he could scarcely have maintained the same percentage absorption when the intake was raised. By taking the differences between the intakes and absorptions on white and brown bread diets, it may be shown that the calcium added as an inorganic salt was equally well absorbed from brown and from white bread diets. The data upon which

TABLE 21. The percentage absorption of calcium from unfortified and fortified diets

Subject	% of dietary calcium absorbed				Calcium added to white bread diets mg./day	% of added calcium absorbed	Calcium added to brown bread diets mg./day	% of added calcium absorbed
	White bread		Brown bread					
	Un-fortified	Fortified	Un-fortified	Fortified				
E. B.	61	39	32	24	530	17	800	19.
N. K.	27	21	5	13	818	18	910	17
R. M.	27	14	3	8	710	2	824	12
P. S.	40	29	4	19	683	19	815	28
B. A.	32	17	9	10	650	9	535	11
A. M.	31	24	15	18	734	20	675	22
E. W.	26	19	9	10	615	14	560	11

these statements are based are given in Table 21. Taken together, these results indicate that brown bread inhibits, as white bread does not, the absorption of small amounts of calcium. The inhibitory effect, however,



is no longer to be observed after a certain level of intake has been reached. These facts are in keeping with the view that phytic acid is the inhibitory agent. This substance, potent though it may be, is limited in its action by its powers of chemical combination. Once these are exceeded, it ceases to exert any harmful effect, and the intestine deals with the excess as though there was no inhibitory agent present at all. It was for some such reason no doubt that Harrison & Mellanby [1939] found that commercial 'Phytin' cured rather than promoted rickets.

*Phosphorus absorption from fortified flour.* The surest way of producing rickets in rats is to feed them on a diet in which the ratio of calcium/phosphorus is very high—at least 3/1. The absolute amount of phosphorus in the diet need not be very low, but the rickets which results is of the low phosphorus type, and the accepted explanation is that the excess of calcium in the gut precipitates the phosphorus there, and prevents its absorption. There is a great deal of evidence in favour of this view, and it was an intimate experience of rickets in rats, rather than in man, which prompted the suggestion that calcium hydrogen phosphate and not calcium carbonate should be used to fortify the nation's bread. It has just been shown that calcium, a shortage of which is the main cause of human rickets, is absorbed equally well from either salt, so that it is unnecessary to fortify with the phosphate if the only object is to increase the absorption of calcium. It may readily be shown that fortification with the phosphate increases the absorption of phosphorus, and the data have already been given in Table 11. Table 22 shows the effect of fortifying the bread with calcium carbonate on the absorption of phosphorus. It will be seen that, whether the bread was white or brown, fortification invariably reduced the percentage of the phosphorus which was absorbed. This is unquestionably the same mechanism which produces rickets in rats, but it is no new observation in human metabolism. Schabad [1910] seems to have been the first to record it as the result of a balance experiment on a single healthy child. Orr, Holt, Wilkins & Boone [1924] made a similar observation on two babies, neither strictly normal, and Bowditch & Bosworth [1917] have also noted it. In the present experiments the absorption of phosphorus was only slightly reduced, and the reduction would never be of any dietetic importance to persons taking brown bread as the basis of their diets, for these contain so much phosphorus that anyone living on them would always have enough and to spare. The reduced absorption on the white bread diets requires a little more consideration, for they originally contain much less phosphorus. The present observations might be used as an

TABLE 22. The effect of calcium carbonate on phosphorus absorption

Subject	White bread				Brown bread			
	Unfortified		Fortified		Unfortified		Fortified	
	P intake mg. day	Absorp- tion % of intake	P intake mg. day	Absorp- tion % of intake	P intake mg. day	Absorp- tion % of intake	P intake mg. day	Absorp- tion % of intake
E. B.	1250	73	1130	69	1970	54	2330	50
N. K.	1270	77	1310	70	2400	55	2620	48
R. M.	1530	59	1400	55	2330	44	2400	41
P. S.	1200	67	1370	61	1650	48	2340	47
B. A.	995	71	1080	63	1850	50	1760	42
A. M.	850	71	1000	65	1660	53	1750	47
E. W.	1120	67	1270	60	2120	55	1950	45
R. W.	990	57	1230	59	1300	47	1650	46

argument against the use of white flour, but among the experimental subjects at least 50% of the dietary phosphorus was always absorbed, even from the fortified diets, and this should have provided for all the requirements of metabolism. Furthermore, in these experiments, 132 mg. of calcium were added to every 100 g. of white flour. Had the smaller quantity recommended by us and by the Medical Research Council [1941] (see below) been added, the effect on phosphorus absorption would have been smaller and probably quite insignificant.

#### IMPLICATIONS OF THIS WORK AT THE PRESENT TIME.

The question of whether calcium should be added to flour at the present time raises some rather difficult issues. They would appear to be: (a) Was the calcium intake of the population of this country deficient before the war? It is impossible to be dogmatic about this because so little is known about the calcium intakes of individual members of the community. There is also the uncertainty about what constitutes a person's calcium requirement—but see below. According to Orr [1937], Khan [1940], and others, many of the population were living below their optimum dietary requirements, although it must be admitted that there was little clinical evidence in adults of calcium deficiencies of an uncomplicated dietary type. Some people can only be convinced by evidence of this nature, but these people are apt to forget that it is not the adults that matter, but the children, and that they matter even before they are born. Judging by the published and unpublished work of this department, many adults and children, even of the middle class, were taking less calcium than the amounts which seem to be desirable. (b) Have the calcium intakes of the people of this country been reduced by the war, and are they likely to be further reduced? The richest

sources of calcium in English diets are milk and cheese, but eggs and green vegetables are also important. The first of these has been restricted so far as adults are concerned, and the second and third have been severely rationed. Obviously the intakes of calcium have been reduced; the extent of their further reduction depends mainly upon the future supplies of milk and cheese. (c) What percentage of the whole wheat grain is to be used for human consumption? We have no official information about the food position, or the cereal reserves of Great Britain. There is evidently opposition in certain quarters to the introduction of a flour of high extraction, but the Government's hand may be forced—just as it was in the last war—and flours of high extraction may become the staple. It may still just be possible to make out a case for not adding calcium salts to white flour. Those afraid or unwilling to take the step can still shelter behind the consolation that the health of the people is not likely to deteriorate much below pre-war standards so long as the milk supply can be maintained. If, however, the public are to be invited to partake of flour containing 92% of the original wheat, then, in our opinion, the Government would be neglecting its duty if it failed to fortify these flours with calcium. (d) Can a moderate excess of calcium do any harm? The answer must surely be—certainly not. If the body were so constituted that it was unable to correct minor excesses in the food supplied to it from time to time, we should none of us be alive to-day. Furthermore, the calcium intakes of the English are far below those of other nations, such as the Finns, whose physique, longevity and health are quite as good as our own.

Reference must be made at this point to a thoughtful article by Nicholls & Nimalasuriya [1939], in which they put forward the view that the human being has such powers of adaptation to a change in his calcium intake that to worry unduly over such a matter is to make much ado about nothing. They point out that Ceylonese children often get no more than 0.2–0.4 g. of calcium per day. Yet they grow and look normal, and their bones resemble those of European children in structure and composition. The short stature of the Ceylonese seems to be the weak point in this argument. The authors themselves give the average female height to be less than 5 ft. and the male 5 ft. 4½ in. Furthermore, Nicholls & Nimalasuriya state that the better class are taller and heavier than their poorer neighbours. The height of these children, therefore, is probably less than that to which they would grow if their calcium intakes and general nutrition were improved.

Turning now to the matter of how much calcium carbonate should be

added to the bread, this clearly depends upon whether the present milk supplies can be maintained, upon how much of the nation's calorie intake will be derived from the staple cereal, and upon what the nature of that cereal is likely to be. Only those with the necessary inside information can make these forecasts. Assuming, however, that conditions will be similar to those created for the present experiment, viz. milk supplies reduced and wheat providing 40-50% of the total calories, then data derived from this study can be used as a guide. In point of fact the experimental conditions were not unlike what the national situation may yet become, so that this is probably as good a way as any other of assessing the amount of calcium to add. Accordingly, all the experimental data have been assembled and inspected, and the results are summarized in Table 23. The experimental periods upon which these figures are based

TABLE 23. The assessment of calcium requirements.

Subject	Absorption needed to maintain Ca balance mg./day	White bread		Brown bread	
		Intake by mouth needed to maintain balance mg./day	Intake by mouth needed to promote + balance mg./day	Intake by mouth needed to maintain balance mg./day	Intake by mouth needed to promote + balance mg./day
Men					
E. B.	169	450	500	530	1420
N. K.	229	1250	1350	1600	>1700
R. M.	214	745	>1400	1500	1600
P. S.	260	644	1330	1530	>1600
C. B.	222	585	—	—	—
Average	218	734	>1140	1290	>1600
Women					
B. A.	146	394	1030	1000	>1100
A. M.	129	415	1140	530	1220
E. W.	119	450	1070	785	1220
R. W.	157	526	> 872	1050	>1100
K. B.	136	500	—	572	—
Average	136	457	>1030	785	>1160

were not all of equal length, and a modicum of judgement has been exercised in their selection and presentation. Exceptional results, for example, have been excluded, and a certain amount of averaging has been done. The men and the women have been separated for the obvious reason that their requirements, both nett and gross, were consistently different. No record has been found of a similar observation, although such a finding has been foreshadowed by the tendency in recent years to express calcium requirements as so many mg. per kg. of body weight. It will be seen from the table that the men needed to absorb about 220 mg.

of calcium per day to maintain themselves 'in balance', the women only 136 mg. Naturally, the individuals differed somewhat among themselves, but there was no overlapping of the sexes. These were the subjects' true, i.e. internal, calcium requirements. A figure for the intake of calcium necessary to maintain balance on a white bread diet was obtained from five men and five women. The figures averaged 734 and 457 mg./day respectively, and these may be said to have been their minimum food requirements. Taking into account the differences in the diets and methods of assessment, these figures agree very well with those of Outhouse, Breiter, Rutherford, Dwight, Mills, & Armstrong [1941], and of Steggerda & Mitchell [1941]. Positive balances were obtained at the figures shown for three men and three women, but one man and one woman failed to retain calcium at their highest experimental intakes. The averages, therefore, for what may be termed these men's and women's luxury food requirements are lower than they ought to be. Assuming the working dietary requirement (or optimum dietary requirement) to be the mean of the figure which maintains the subjects in balance, and the one which promotes calcium storage, then the optimum calcium requirement on white bread diets was 947 mg./day for the men of the experimental party, and 744 mg./day for the women.

Turning to the brown bread diets, it is obvious that much higher intakes were required to maintain calcium equilibrium. The average figure for minimum food requirement was 1300 mg./day for the men and 785 mg./day for the women. Positive balances were obtained from so few of the subjects on the brown bread diets that it was impossible to form any estimate of the luxury requirements of the party by averaging the limited data. Working therefore from the minimum/luxury ratios for A. M. and E. W. on brown bread diets, and from the minimum/luxury ratios for the whole party on white bread diets, a figure of 2100 mg./day has been arrived at for the men's luxury requirement on brown bread diets and one of 1400 mg./day for the women's. Taking as before the optimum dietary requirement to lie midway between the minimum and the luxury requirement, then the optimum requirement on brown bread diets was about 1700 mg./day for the men and 1100 mg./day for the women.

Had 65 mg. of calcium been added to every 100 g. of white flour, and 200 mg. to every 100 g. of 92% flour, these requirements would have just about been met. To persons eating 1 lb. of white or brown bread per day, these additions of calcium would be no more than the amounts in  $\frac{1}{3}$  or  $\frac{9}{10}$  of a pint of milk respectively.

In all fairness it must be stated that this method of assessing requirements has been criticized. Nicholls & Nimalasuriya [1939] state: 'If a person is accustomed to a calcium intake of 0.6 g. daily his metabolic processes will become adapted to this amount.... If suddenly his intake is reduced to 0.3 g., his metabolism will not be adapted to this amount for some time, and consequently this intake will be followed by negative balances.... Provided the calcium balances of an individual are determined over a sufficiently long period of time, all that such experiments will reveal is the daily amount of calcium the individual is accustomed to take; and calculation from these of optimum requirements cannot be made.'

This criticism can be met from the present results themselves. The experimental diets contained less calcium than those to which the subjects had been accustomed. Yet their calcium balances were not uniformly negative at the beginning, nor did any of the subjects show any signs of adaptation over a period of 9 months. In fact some of their absorptions showed every sign of deterioration, not improvement, as the time passed and the low intakes were maintained.

There is another way of assessing how much more calcium should be added to 92 than to 69 % flour. Assuming that the breads are baked with yeast in the usual way, and the normal amount of phytate is destroyed in the process, 158 mg. of calcium would have to be added to every 100 g. of 92 % flour to precipitate all the phytic acid in it as the calcium salt. There is so little phytic acid in white bread that it may be considered for this rough calculation to contain none. 100 g. of brown bread therefore might require to contain 158 mg. more calcium than white before it became metabolically equivalent. This is in very fair agreement with the figure of 135 mg. derived from the balance experiments, for some of the phytic acid in the brown bread would certainly be precipitated as the magnesium salt, and hence the figure of 158 is somewhat too high.

When these experiments were planned the Ministry of Food had not yet given birth to the National Wheatmeal Loaf. It is obvious that if 69 % flour requires to be fortified in the interests of general health, 85 % flour must be fortified all the more. It stands midway between the 69 and 92 % flours so far as its phytic acid is concerned. Assuming that phytic acid is the main agent in all these flours which inhibits calcium absorption, and knowing that it is destroyed in baking bread with 85 % flour, as it is in baking with 69 and 92 % flours [Widdowson, 1941], then 120 mg. of calcium added to every 100 g. of 85 % flour should just about have met

the optimum calcium requirements of the experimental subjects, had they been tried on such a diet.

It may be recommended, therefore, that 65 mg. of calcium be added to every 100 g. of 69% flour to be used in this country during the war, and that 120 mg. should be added to 100 g. of the National 85% flour. It may further be suggested that if it is at any time desirable to introduce a 92% flour for general use, 200 mg. of calcium should be added to every 100 g.

In making these recommendations, it seems wise at the same time to sound a note of warning. The Ministry of Food thinks—and must think—in terms of national requirements. If the nation is short of calcium because its milk supplies have been curtailed, the simplest way of putting this right (from the Government's point of view) is to add calcium to bread—because everyone eats bread. But people will not change their dietary habits to please the Ministry of Food. The large milk consumer may—or may not—become the big bread eater when his favourite beverage is restricted. Hence, many who were well supplied with calcium may go short, and, vice versa, many who were taking very little may suddenly find themselves with more than they had before. It is clear, therefore, that although the addition of calcium to bread may maintain the nation's calcium intake at its previous level, there will be a great deal of watchful care needed on the part of those responsible for the well-being of individuals.

There is one further point to be mentioned. Unfortified brown bread depresses the absorption of calcium. The more one eats, the worse one's calcium balance is likely to become. If phytic acid is the main inhibitory agent, and just enough calcium has been added to inactivate it, the bread may be described as neutral, and the calcium balances should not be affected by the amount of bread consumed. If more than enough calcium has been added to precipitate all the phytate, the bread will supply free calcium available for absorption, and balances are likely to improve as consumption rises. White bread contains so little phytate that the unfortified material is probably fairly neutral, and any degree of fortification would probably make the bread a source of available calcium.

#### SUMMARY

1. Balance experiments have been carried out over a period of 9 months on five healthy men and the same number of women.

2. The absorption and excretion of minerals have been studied when 40-50% of the calories in these subjects' diets were provided by wheat

flours of the following types: 69% extraction; 92% extraction; 69% extraction fortified with calcium carbonate or mono-hydrogen phosphate; 92% extraction fortified with the same salts; 69% extraction with the addition of sodium phytate; 92% extraction with a supplement of 2000 I.U. of calciferol per day.

3. The following conclusions have been reached:

(a) The calcium, magnesium, phosphorus and potassium in diets made up with 92% flour were less completely absorbed than the same minerals in diets made up with 69% flour. Hence in defining calcium requirements it is essential to state the nature of the cereal in the diets.

(b) Sodium phytate added to 69% flour depressed the absorption of calcium and magnesium, but not of potassium. About 50% of the phosphorus in sodium phytate was absorbed.

(c) Vitamin D did not materially improve the absorption of calcium from diets made up with 92% flour.

(d) Fortifying the bread with calcium salts improved the absorptions of calcium, and prevented a loss of calcium from the body if this had been taking place. The carbonate and phosphate were equally efficacious. The addition of calcium carbonate slightly depressed the absorption of phosphorus.

(e) It has been recommended that flours for national use during the present emergency should have calcium carbonate added to them in the following proportions: white flour, 65 mg. of calcium per 100 g.; National 85% wheatmeal, 120 mg. of calcium per 100 g.; 92% wheatmeal, 200 mg. of calcium per 100 g.

The present work really represents the united efforts of the ten subjects, B. A., C. B., K. B., E. B., N. K., A. M., R. M., P. S., E. W. and R. W. The authors have merely had the pleasant task of committing the results to paper. The Medical Research Council made the investigation possible by their financial help.

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# DIFFUSION RELATIONS OF UREA, INULIN AND CHLORIDE IN SOME MAMMALIAN TISSUES<sup>1</sup>.

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For a substance contained in the fluid external to a tissue, the equilibrium amount per 100 g. tissue divided by the amount per 1 ml. of fluid gives a measure of the tissue 'space' in which the substance is dissolved, provided that at the same time it is neither appreciably adsorbed nor concentrated within. If instead of the amount per 100 g. tissue we consider that in 100 g. of tissue water, divided as before by the amount per ml. external fluid, we have the value already defined as a 'permeation' [Conway & Cruess-Callaghan, 1937]. Such a quantity, which can be treated quite empirically and at the outset as only numerically descriptive, is useful, for example, in comparing the equilibrium values of urea dissolved in tissues. Usually a 'permeation' of about 100 may be expected. If definitely higher than 100 it indicates that urea is being formed in appreciable amounts as in the liver, or is being concentrated for excretion, as shown with suitable controls for the isolated kidney of the frog [Conway & Kane, 1935], or again is being adsorbed on surfaces. If lower than 100 it shows that some tissue water is not free for solution or that there is a region impermeable to urea. Urea has already been used to investigate the 'free water' for solution in muscle [e.g. Eggleton, 1930], a question dealt with at some length in a previous paper from this laboratory [Boyle & Conway, 1941]. Here we are concerned rather with comparisons between tissues and with respect to inulin, chloride and urea, and use the 'permeation' values throughout. These can be readily converted to 'spaces' in the usual sense by multiplying by the tissue water fraction.

<sup>1</sup> A preliminary account of some of the findings in the present paper (including the use of inulin to measure the intercellular spaces) has been already presented by one of us in thesis form [FitzGerald, 1937].

The 'permeation' of inulin or alternatively the inulin 'space' may be expected to give a measure of the free intercellular spaces. It has been used here to determine the spaces in the isolated sartorius muscle of the frog [Boyle, Conway, Kane & O'Reilly, 1941] and by McCance [1938] to determine the total intercellular spaces in the human subject after intravenous injection.

Where the inulin 'permeation' considerably exceeds any probable estimate of the intercellular spaces, and provided we eliminate the possibility of an adsorption explanation, as by comparison with other tissues, we may conclude that the cells are in part at least permeable to inulin. Again, if the inulin 'permeation' is definitely less than the chloride value it may be assumed that chloride is either adsorbed on the surface of the tissue elements or is present within them, or probably distributed in both these ways. Such considerations are theoretically important for muscle, renal and nerve tissue.

Diffusion coefficients for urea and inulin have also been determined for renal cortical sections, and also for urea in muscle, liver and brain tissues. Chloride data have been used only for 'permeation' comparisons with urea and inulin in muscle and nerve tissue.

Throughout we have used concentrations in plasma as if the volume of this could be regarded as entirely free water for solution. This, of course, is not strictly correct, and would involve small reductions in the 'permeations' as given. For such plasma values, however, we have considered it advisable to keep to the conventional usage.

## METHODS

*Experimental.* Urea 'permeations' were measured simply from analyses of tissue samples and of blood plasma taken at the same time. This was varied by raising the blood urea with intravenous injections of 5% urea in 0.6% NaCl (10–40 ml./kg.), carried out usually under ether anaesthesia. For renal tissue this procedure is not applicable, and here, in order to remove the lumen concentration, a cannula was very quickly introduced into the renal artery or the aorta, and the freshly excised kidney was perfused with a solution recommended by Krebs & Henseleit [1932] but containing 0.01–0.02 M cyanide, the gas mixture being either 5% CO<sub>2</sub> with 95% O<sub>2</sub> or 95% N. The perfusion was conducted at 37° C. under a pressure from the above gas mixture of about 100–160 mm. Hg, the external surface of the kidney being also maintained at about 37° C. The perfusion was usually carried out for about 15 min., about 200 ml. fluid being sent through. It was shown that under these

conditions the cyanide removed any appreciable concentrating effect in the flowing urine, and that with a urea-free solution all the urea in the kidney was washed out after the first few minutes. When the perfusing fluid contained 0.2 % urea the 'permeation' was obtained by determining the renal cortical concentration after perfusion. Alternatively, after perfusing with a urea-free solution the cortex was sectioned, the sections being allowed to diffuse in a urea-Krebs fluid, 30-60 min. being quite sufficient time. The diffusion chambers used are described below.

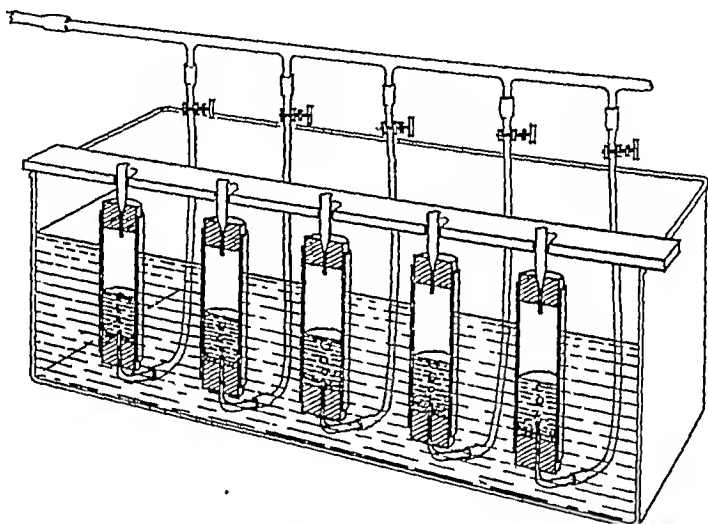


Fig. 1. Sectional view of diffusion chambers in thermostat. See text.

Inulin permeations were determined similarly after intravenous injections of 0.6 % NaCl with 1 % inulin (50 ml./kg.). For the renal cortex the procedure was similar to that for urea, but cortical sections were also diffused without preliminary perfusion of the kidney. Tissue sections were prepared by the method of Warburg [1930] from animals killed by a sharp blow on the cervical spine, no anaesthetic being used. The sections were not trimmed until after the diffusion, the area being then determined by using trans-illuminated squared paper. From this and the weight of the trimmed tissue a mean value for the thickness could be obtained. The mean thickness of a large series was found to be 0.7 mm., but cortical brain sections could not be obtained finer than 1.0 mm., and for muscle tissue it was found preferable to use the whole diaphragm of the rat (mean thickness of 0.9 mm.).

*Diffusion chambers.* Diffusion was conducted in chambers made out of simple pyrex tubes  $8 \times 2$  cm. The bottom of each chamber was formed by a rubber cork sealed with an internal layer of a high-melting paraffin, through which passed a finely pointed tube. Through this latter the appropriate gas mixture was introduced serving also to mix the solution. The arrangement of a series of chambers is shown in Fig. 1.

### *Chemical methods*

Urea was determined in the various fluids and tissues by the improved micro-diffusion technique [Conway, 1939].

*Inulin.* In determining the inulin content of tissues, the principle used was to determine the reducing power (as glucose) of a protein-free extract of the tissue before and after hydrolysis with  $0.1N$  HCl. The results expressed as glucose were then converted to inulin by multiplying by 0.9.

The cadmium sulphate solutions as described by Van Slyke, Hiller & Miller [1935] were used as protein precipitants.

Tissue extracts were made by grinding the tissue (50–150 mg.) with 3.0 ml. water with the aid of a little pure quartz sand (Merck's) transferring with some washing to a small conical flask (25 ml.) and heating momentarily to boiling, cooling, and adding sufficient excess of the strong  $CdSO_4$  solution to precipitate all proteins and then sufficient  $N$  NaOH to neutralize the excess. The mixture was then made up to 5 or 10 ml. and centrifuged. Aliquot volumes made up to 5 ml. were taken for subsequent analysis by a modification of the Hagedorn-Jensen method, after hydrolysing at  $100^\circ C$ . with 0.5 ml.  $N$  HCl added. A similar determination was carried out without hydrolysing for the free glucose or reducing substances present. Subtracting this value from the hydrolysed sample gave gross values for the inulin. Such values are too high, since without any inulin present a slight increase in reducing substance occurs on heating with acid. An allowance for this, as measured from many determinations on different tissues, is sufficiently small and constant to obviate the need of special determinations for each animal. Expressed as mg. glucose/100 g. tissue it amounts to 21 for the kidney (28 determinations), 25 for muscles (4 observations) and 24 for the cerebral cortex (4 observations).

The above method was checked by recoveries of added inulin (Pfanstiehl's used throughout) to the macerated tissue mixtures. These gave a mean value of 99% of the added quantity.

The inulin in the diffusing and perfusing fluids was determined in a similar manner, the protein being directly precipitated with the weak  $CdSO_4$  solution.

*Chloride.* For tissues, this was determined both by the micro-diffusion method [Conway, 1935] and the open Carius method after Eisenman's modification of the Van Slyke procedure [1929]. Blood chloride (plasma) was determined by the micro-diffusion method. This latter method, when applied to rabbit muscle determinations and under the given conditions, requires longer times for the liberation of the free chlorine than that already considered. (This point is being further investigated.)

*Water content of tissues.* As the 'permeation' values are given in terms of the water content, it was necessary to determine this value for the special conditions used. In the procedure used the loss of weight per 100 g. of tissue dried for 12 hr. at  $105^\circ C$ . in a platinum crucible was reckoned as the water content. In this way the water content of the rabbit's kidney was found as 76.4; after perfusing with cyanide-Krebs's solution it was 85.6. For renal sections diffused in Krebs's fluid it was 80.0, and in cyanide-Krebs's fluid 81.8. For

muscle Hill & Kupalow's [1930] value of 80.0 for the total water content (similar to Katz's figure [1896] of 76.8) was taken, and for the brain, Matsumoto's [1933] figures for the rabbit's brain—69.4 and 81.8 for white and grey tissue—were used.

*Haemoglobin.* This was determined in arterial blood samples by the Barker method as modified for spectrophotometric use by Heilmeyer [1933].

### INULIN 'PERMEATIONS'

*Skeletal muscle.* The 'permeations' were determined for rabbit muscle after injecting intravenously 50 ml./kg. of 10 % inulin in 0.6 % NaCl into the animal anaesthetized with ether and allowing at least 30 min. for equilibrium between plasma and muscle. The blood was then removed through a carotid cannula and immediately centrifuged, the plasma being taken for analysis. Simultaneously with the removal of blood, samples of skeletal muscle were excised, weighed and analysed for inulin as described. The results for four rabbits are given in Table 1. The mean

TABLE 1

Rabbit	Conc. in plasma %	Tissue	No. of observations	Range of 'permeations'	Mean 'permeation'
Inulin permeations					
1	1.50	Skeletal muscle	7	9.7- 6.0	7.9
2	1.56	"	4	16.2- 8.1	11.8
3	1.01	"	4	7.7- 4.6	6.1
4	(1.36)	"	7	2.0-15.7	7.9
2	1.56	Cerebral cortex	4	4.4- 2.0	3.0
3	1.01	"	3	0.2- 1.7	0.8
Chloride permeations (similar conditions)					
5	0.601	Skeletal muscle	2	—	12.8
6	0.582	"	2	—	16.5
7	0.576	"	2	—	16.1
8	0.590	"	2	—	16.7
9	0.573	"	2	—	12.8
10	0.574	"	2	—	16.3
8	0.590	Cerebral cortex	1	—	53.8
9	0.573	"	1	—	45.2
10	0.574	"	1	—	49.6
9	0.573	Cord	1	—	59.2
10	0.574	"	1	—	58.3

inulin 'permeation' for the three animals for which plasma figures were available is 8.6 (or an inulin 'space' of 7 %), or  $8.5 \pm 0.6$  for the whole fifteen observations (giving standard error of mean). Thirty minutes would appear to be sufficient for inulin equilibration across the capillaries, if we are to judge by the fact that no appreciable change occurs in the plasma inulin after 30 min. (see Fig. 2). It is true that the volume of the circulation may be decreasing after this time and a loss of inulin be obscured. Determinations of blood haemoglobin carried out on two rabbits similarly treated with inulin injections showed no appreciable

increase from 30 to 120 min., but rather a small decrease, as shown in Fig. 2. From this it would appear reasonable to suppose that no appreciable inulin was *leaving* the capillaries 30 min. after the injection.

Over 30–120 min. after injection the mean plasma inulin concentration was 2.7 g./100 ml. Since 5 g. inulin per kg. were injected, then in 1 kg. rabbit there were  $5/2.7 \times 100$  ml. intercellular space, hence 18.6% of the weight of the animal. Since the fluid in which the inulin was injected was 0.6% NaCl it may be presumed that the water in about

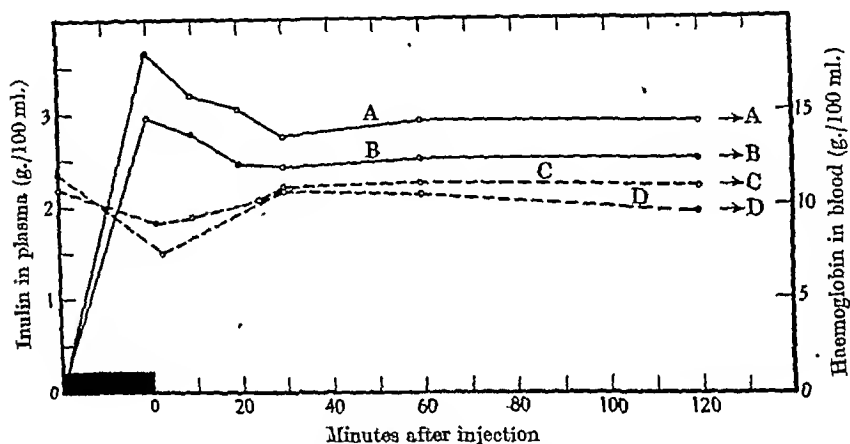


Fig. 2. A and B curves represent inulin concentrations in plasma of two rabbits after intravenous injection of 5 g. inulin/kg. as a 10% solution in 0.6% NaCl. The times of sampling begin from the end of the injection, which took about 20 min. C and D give haemoglobin concentrations in the blood of two rabbits similarly treated. ████████ represents continuous injection of 10% inulin solution in saline.

one-third its volume passed into the tissue cells and two-thirds (3.3% body weight) remained to swell the intercellular fluid. Consequently the original intercellular fluid would have been 15.3% of the body weight, which may be compared with McCance's figure of 15.7% for the human subject [1938].

*Comparison with chloride 'permeation'.* This value of 8.5% for the inulin 'permeation' we may take as giving a maximum value for the interspace water (as a percentage of the total water) in the excised skeletal muscle of the rabbit, and it is very appreciably less than the generally accepted value derived from chloride analyses (e.g. Fenn [1936] for references). The mean chloride 'permeation' was determined for a further six rabbits, using the leg muscles with duplicate determinations and was found, under the same conditions, to be  $15.2 \pm 0.7$  (being some-



what lower than for similar observations on frog muscle [Conway & Kane, 1934; Boyle *et al.* 1941]. The difference between the mean inulin and chloride 'permeations' as measured for the excised rabbit muscle is therefore  $6.7 \pm 0.9$ .

*Renal cortex. Cyanide perfused kidney (rabbit).* The freshly excised kidney of a rabbit was perfused for 15 min. with a cyanide-inulin-Krebs's solution as described in the section on methods. The cyanide content was  $N/200$ , and the inulin 1.0%, no appreciable concentration of inulin occurring in the urine. The results are given in Table 2, six duplicate determinations being carried out with two rabbits. The mean value of the inulin permeation was 56.5% with a range of 42.6-63.8.

TABLE 2

Tissue	Inulin concn. in perfusate	Inulin 'permeations'	Mean inulin 'permeations'
Renal cortex	1.0	56.6; 42.6; 47.7	49.0
Renal cortex	1.0	63.8; 63.0; 62.5	63.1

*Renal cortex. Sections diffused in inulin solution.* Sections from the kidneys of rabbits which had received no injections were introduced as quickly as possible into the diffusion chambers containing Krebs's solution with 1% inulin stirred with the gas mixture as described. They were removed at varying times, dried between filter paper, rapidly but carefully, so that all adherent fluid was well removed. Table 3 gives a

TABLE 3

External inulin conc. g./100 ml.	No. of sections analysed	Mean time of immersion of renal sections min.	Mean thickness of tissues mm.	Mean 'permeation'
0.900	2	45	0.9	29.8
9.670	2	45	0.7	56.3
0.900	2	52	0.7	25.0
0.800	3	61	0.6	47.5
0.800	3	65	0.7	30.2
0.800	3	70	0.7	38.0
0.900	2	120	0.8	51.7
0.900	2	180	0.8	53.2

summary of the results obtained from experiments on seven animals. The total average 'permeation' (nineteen sections) was 41.5% or five times as great as that found for muscle tissue. After 2-3 hr. it was 52.4%. The majority of these sections were analysed for inulin without using the momentary boiling of the ground tissue extract, and may have given values somewhat under the true figures.

*Cerebral cortex.* Inulin 'permeations' determined out on two of the rabbits examined for inulin 'permeations' in muscle, the results being extremely low (Table 1), ranging from 0.2 to 4.4% with a mean of 1.9%.

Chloride analyses carried out under similar conditions give 'permeations' ranging from 45.2 to 53.8 for the cerebral cortex and 59.2 and 66.3 were found for the cord.

#### UREA PERMEATIONS

*Skeletal muscle.* Determinations were carried out on two normal rabbits and two rabbits and two rats after intravenous injections of urea, 40 ml./kg. of 5% urea in 0.6% NaCl. The mean value of the 'permeation' in eleven determinations was 93% when 80% of the tissue weight was taken as that of the total water present [Hill & Kupalow, 1930].

*Renal cortex. Cyanide perfused kidneys.* The kidney was perfused as for the inulin determinations, the perfusing fluid containing 0.2% urea. The mean of nineteen determinations on six cats and two dogs was 94.4% with a standard deviation of the mean of 2.1%.

TABLE 4

Tissue	No. of animals (no. of estimations in brackets)	Time after perfusion or immersion min.	Conditions	Mean conc. of urea in blood plasma or ext. fluid %	Mean 'permeation' (with s.d. of mean)
al cortex	6 cats (19) 2 dogs	20	Perf. with Krebs's fluid containing 0.01% NaCN	0.2	94 ± 2.1
al cortex	6 cats (25) 1 dog	30-60	Sections diffused in Krebs's solution containing urea and cyanide	0.03-0.2	107 ± 3.2
al cortex	6 cats (23) 1 dog	30-60	Sections diffused in Krebs's solution with urea and no cyanide	0.2	109 ± 2.8
keletal muscle	3 rats 2 rabbits	5-60	Intrav. inj. of 10-40 ml. 5% urea (in 0.6% NaCl) per kg.	0.479	92.9 ± 4.0
rebral cortex	2 rabbits 2 rabbits	30	No injections Inj. of 40 ml. 5% urea (in 0.6% NaCl) per kg.	0.061 0.363	86.5 34.6
rebral cortex	2 rabbits	60	Inj. as above	0.368	35.1
rebral cortex	1 rabbit	270	Inj. as above	0.344	62.0
rebral cortex	1 cat	25	Inj. as above	0.535	44.0
ord	2 rabbits		No. inj.	0.061	63.0
ord	2 rabbits	60	Inj. of 40 ml. 5% urea (in 0.6% NaCl)	0.385	32.8
ord	1 cat	25	Inj. as above	0.535	15.0

*Renal cortex. Diffused sections (30-60 min. in chambers).* For six cats and one dog the following procedure was used. The kidneys were perfused for 15-20 min. immediately after excision and as described under Methods with a cyanide-urea solution (0.2% urea and *N*/200 NaCN). Sections were made of the renal cortex, some of which were

taken for immediate analysis, some transferred to a diffusion chamber containing 0.2 % urea without cyanide and others into a diffusion chamber containing a similar fluid to that used in perfusing. The sections in the chambers were allowed to diffuse for 30-60 min. being then dried and analysed for urea. The results are shown in Table 4 and in the histograms of Fig. 3.

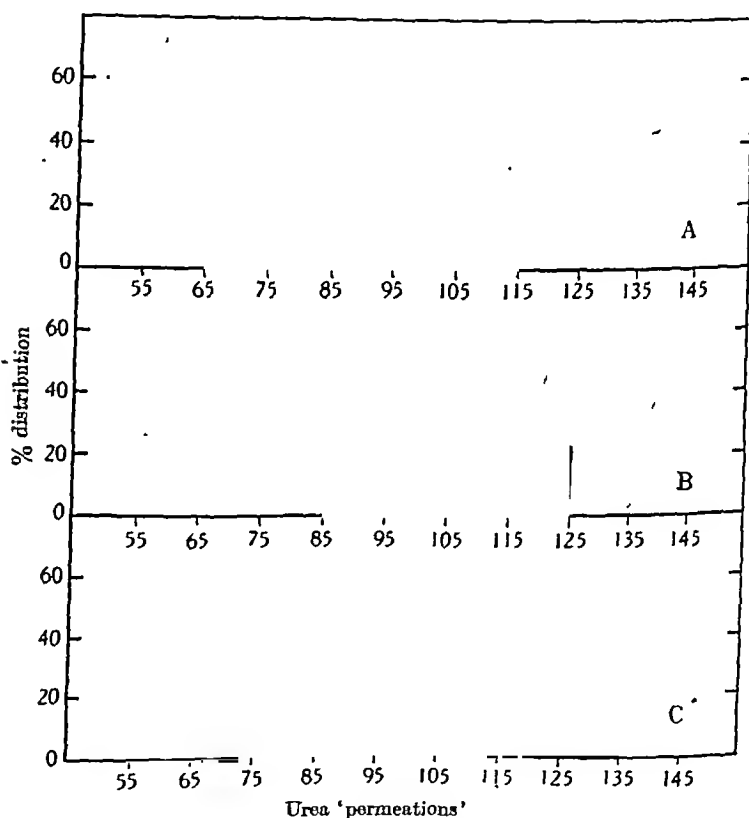


Fig. 3. Urea 'permeation' for renal cortical sections from six cats and one dog, and for which there are corresponding figures for sections (A) immediately after the cyanide-urea perfusion of whole kidney, and (B) after subsequent diffusion of sections in the same fluid as used for perfusion and (C) after diffusion as in (B), but without cyanide.

The mean value after perfusion (nineteen sections) was  $94.4 \pm 2.1$ . After a subsequent diffusion of twenty-five sections in cyanide urea it was  $107 \pm 3.2$  and in urea solutions without cyanide it was  $109 \pm 2.8$ . The significance of these results is considered in the discussion.

*Cerebral cortex, medulla and spinal cord.* Without injections the mean urea 'permeations' of the cerebral cortex and cord of two rabbits were 86.5 and 67 % respectively. Half an hour after the intravenous injection of 40 ml./kg. of 5 % urea in 0.6 % NaCl the 'permeation' for the cerebral cortex was 34.6 %, the plasma being 0.363 g./100 ml.; 1 hr. after injection the value was 36.6 % for three rabbits; and 270 min. afterwards it was 62 % (one rabbit). The 'permeations' for the cord as observed 1 hr. after the urea injection were similar to the cerebral cortex. As shown in Table 4 the results for the cat (one animal) resemble those for the rabbit.

### DIFFUSION COEFFICIENTS

#### *Diffusion coefficients through an agar gel at 38° C.*

An agar gel (2.3 %) was prepared containing 2.0 % inulin and 0.5 % urea. This gel was allowed to settle upright in Hagedorn-Jensen tubes at 38° C. When solid the tubes were placed in a water-bath for some

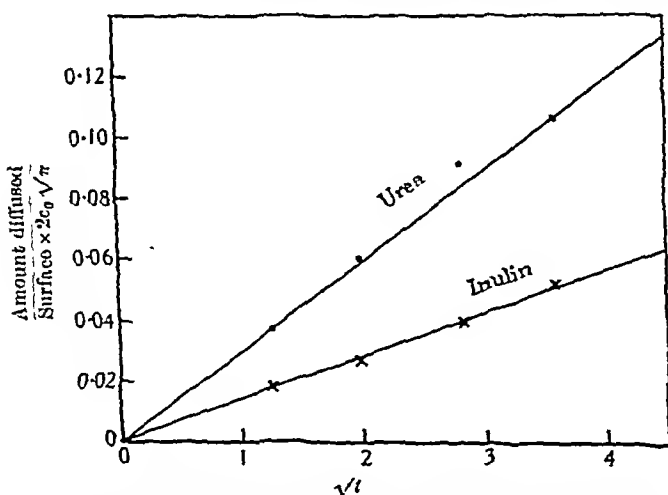


Fig. 4. Diffusion of urea and of inulin through an agar gel. The slopes of the curves give the square roots of the diffusion coefficients.

time to allow for temperature equilibration. Then at a noted time 10 ml. of Krebs's fluid at 38° C. was run over the surface and stirred by bubbling with the 5 % CO<sub>2</sub>-oxygen mixture. Samples were removed for analysis of inulin and urea at different times, and a graph plotted of amounts diffused against  $\sqrt{t}$ . The diffusion coefficients (cm.<sup>2</sup>/min.) were determined from the slopes of the lines in Fig. 4, in accordance with the equation

$$\frac{\text{Amount diffused}}{\text{Surface}} = 2c_0 \sqrt{\frac{Dt}{\pi}}$$

The value for inulin was found as  $19.6 \times 10^{-5}$  and for urea as  $88.0 \times 10^{-5}$  (cm.<sup>2</sup>/min.). These figures are of a similar order to those found by Bunim, Smith & Smith [1937], using a somewhat different method.

### *Diffusion coefficients in mammalian tissues*

*Urea.* The general procedure consisted in diffusing sections of tissues, prepared immediately after killing the animal, either in a Krebs's solution containing urea or in one free from urea, and determining the urea concentration in the tissues after a definite time. The diffusion coefficient is then calculated in accordance with the following formula [Conway & Kane, 1934]:

$$\frac{\text{Amount diffused}}{\text{Surface}} = 2 (ec_0 - c_1) \sqrt{\frac{kt}{\pi}},$$

where  $c_0$  is the concentration in the tissue,  $c_1$  in the external fluid and  $e$  an equilibrium factor. Since the urea in the external solution will be in simple equilibrium with the urea in the tissue water and since the tissue water is 76 g./100 g. of tissue or 0.81 ml./1 ml. tissue (sp. gr. being taken as 1.05),  $e$  will therefore be 0.81. (If this equilibrium factor be neglected the observed diffusion coefficient for urea diffusion *inwards* will be only 0.66 of the value *outwards*, and for other substances the ratio may be as low as 0.05 or less.)

Sections of the renal cortex were diffused under a variety of conditions, i.e. either directly in cyanide-Krebs's fluid or after an initial perfusion of the kidney with or without cyanide. For skeletal muscle the diaphragm of the rat was used (mean thickness of 0.9 mm.), it being realized at the same time that mammalian skeletal muscle swells in the usual 'isotonic' fluid.

The results are summarized in Table 5. The mean values of the diffusion coefficients for different animals and tissues range from  $5.3$  to  $12.3 \times 10^{-5}$  with a general mean of  $8.65 \times 10^{-5}$ . The observations on single sections show considerable variation as shown in the table. The main source of this variation is apparent when the diffusion coefficients are graphed against the section thickness (omitting observations on cerebral cortex and cord which appear different in kind). From Fig. 5 it is obvious that increasing thickness in section causes an increase in the diffusion coefficient, the correlation between  $d$  and  $k$  being 0.78. From the line of best fit the mean coefficient of  $2.5 \times 10^{-5}$  was found at a section depth of 0.5 mm., an extrapolation of the curve beyond this point being scarcely permissible from the data. These data, it may also be noted, are mainly from the renal cortical sections. From Fig. 5 it

TABLE 5

Tissue	Animal	No. of animals	No. of sections	Mean depth of sections	Range of $k$ ( $\times 10^3$ )	Mean $k$ ( $\times 10^3$ )
Urea diffusion coefficients						
Kidney	Rabbit	4	14	0.76	0.5 - 10.4	5.3
	Cat	1	2	0.77	6.7 - 7.8	7.3
	Dog	2	10	1.14	2.7 - 21.2	12.3
Muscle	Rat	2	3	0.85	7.5 - 10.3	8.2
Liver	Rabbit	1	1	1.11	—	7.3
	Rat	2	3	1.13	7.6 - 13.2	11.2
Cerebral cortex	Rabbit	2	2	1.56	4.22 - 5.9	5.1
	Cat	1	1	1.29	—	11.8
Spinal cord	Rabbit	2	2	2.42	6.5 - 80	7.2
Inulin diffusion coefficients						
Kidney	Rabbit	1	3	1.11	3.6 - 3.8	3.8
Cortex	Rabbit	1	4	0.88	2.3 - 4.3	3.1

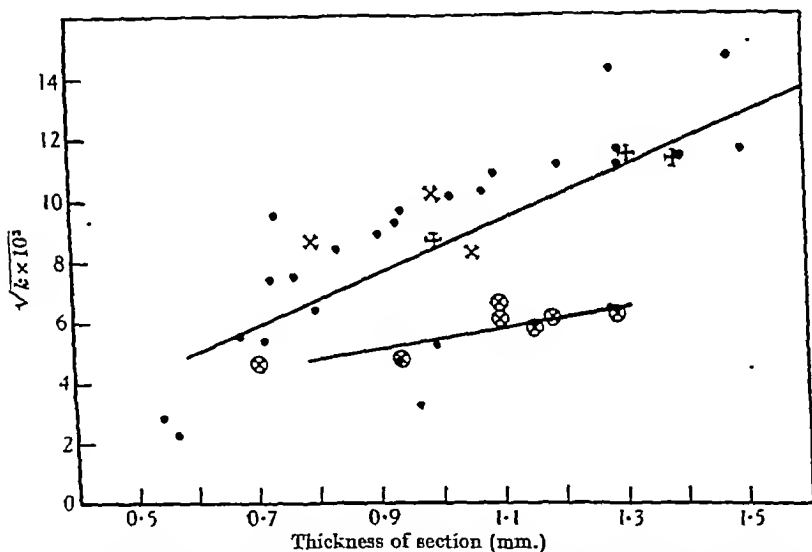


Fig. 5. Influence of section thickness on diffusion coefficients of urea through renal cortex (dots), diaphragm muscle (x) and liver (+); also on the diffusion coefficient of inulin through renal cortical sections (circles with crosses).

appears also that the values for muscle and liver fall into the distribution of renal values and the mean coefficients for these tissues with large numbers of data will probably be very similar to that for the renal cortex. Why the thickness of the section should affect the diffusion coefficient is not immediately obvious since when the bulk of the sections were diffused in a cyanide-Krebs fluid, oxygen lack was scarcely operative; yet the

effect is probably associated with a higher gradient of metabolites in the thicker sections.

*Inulin.* Inulin diffusion coefficients were determined only with renal sections, which gave a relatively high 'permeation' value. The mean for seven sections from two rabbits was  $3.34 \times 10^{-5}$  (range 2.2-4.6). These are indicated in Fig. 5 (circles with crosses) and show also—though few in number—a similar effect of section thickness.

### DISCUSSION

If, for a given tissue, we consider the 'permeations' of different substances injected intravenously, the lowest sets a maximum value for the intercellular fluid continuous with the plasma, provided it can pass the capillary walls and that a sufficient time is allowed for equilibration with the blood concentration.

For most tissues we may expect inulin to be a very suitable substance for determining the intercellular spaces. From the data considered in the results after inulin injection, it is clear that it passes freely across the capillary walls (Fig. 2) and would appear to have reached equilibration in about half an hour. Thirty minutes after injection the inulin 'permeation' for excised leg muscle is  $8.5 \pm 0.6$  and for chloride  $15.2 \pm 0.7$ , the difference being  $6.7 \pm 0.9$ , so that the mean difference for a very large number of observations may be taken as lying somewhere between 4 and 9 (taking  $\pm 3$  times the standard error of the mean). These figures may be compared with the inulin 'permeation' for the isolated sartorius muscle of the frog immersed in an inulin-Ringer fluid [Boyle *et al.* 1941] which is 11-12% (or 9-10 as a 'space'), the chloride figure being 17.6 (this latter figure referring to the freshly excised muscle and the corresponding plasma value).

Considering how the difference between the inulin and chloride 'permeations' is made up in the case of rabbit muscle, we have first the question of the circulatory space and the red corpuscles therein, which though containing chloride may be presumed to contain no inulin. For the isolated sartorius of the frog it was shown [Boyle *et al.* 1941] that the circulatory space is only about 2% and a similar determination for freshly excised rabbit muscle has given a figure of 2% as a maximum value (i.e. neglecting the error arising from the muscle haemoglobin). Only about 1% at most can therefore be assigned as arising from the chloride and inulin difference in the red corpuscles.

The major part of the difference between the inulin and chloride 'permeations' arises from the presence of some chloride within the fibres

and related to the potassium concentration by an equality of the products of these ion concentrations on each side of the membrane [Boyle & Conway, 1941]. For the estimation of this chloride fraction we have the following data:

K in muscle and in plasma	97.4 and 5.5 m. equiv./kg.
Cl in plasma	97.6 m. equiv./kg.
Water in muscle and in plasma	0.8 and 0.926 l./kg.

(The figure for potassium in rabbit muscle is from Costantino's data [1911], and the plasma potassium and plasma water from Abderhalden's figures [1909].) For the calculation the interfibre volume may be taken as 0.1 l./kg. (or approximately 10% of the weight, but a few per cent more or less will here have no appreciable effect). In such intercellular space there will be 0.5 m. equiv. potassium/kg. muscle so that 96.9 m. equiv. potassium will exist in the fibres and in 0.7 l. of water. The concentration of potassium in the fibre water is therefore 138 m. equiv./l. The product of the potassium and chloride concentrations in plasma water is 626, so that the concentration of chloride in the fibre water must be  $626/138$  ( $=4.6$ ) if the Donnan relation applies. This would give  $4.6 \times 0.7$  or 3.2 m. equiv./kg. muscle. This divided by the external plasma concentration and by 0.8 l. for the total muscle water gives a 'permeation' allowance of 4.2. Adding to this a red corpuscle allowance of 1.0 we obtain finally 5 as the approximate expected difference between the inulin and chloride 'permeations'. The figure found is  $6.7 \pm 0.9$ , which differs from 5 by no more than may be expected from the sampling error. It would seem unnecessary therefore to consider with such data further causes for the increase in the chloride 'permeations' over the inulin value for rabbit muscle.

*Inulin 'permeation' of the renal cortex.* The best values for the renal cortical 'permeation' gave figures of over 50% or about seven times more than for muscle and 25 times more than for the cerebral cortex. The total average for the diffused sections was 41.5.

It is unlikely that so high a figure represents merely free space for diffusion or intercellular space. It is true that some increase in water content occurred as judged by the increase in water proportion of the tissue sections from 76.4 to 79.4% and that the conditions were abnormal, but a practically identical figure is given for excised frog kidneys (as shown in experiments it is hoped to describe later). These kidneys lose weight instead of gaining it in Ringer fluid isosmotic with the blood, and are demonstrably active. Further, the haemoglobin 'permeation'



determined at the same time in such experiments is much lower than the inulin 'permeation'.

*Urea 'permeations' in the renal cortex and evidence for active excretion.*

It was shown [Conway & Kane, 1935] for the isolated kidney of the frog that all the urea was freely diffusible therefrom into an external Ringer fluid, and that it re-entered the kidney immersed in cyanide-Ringer fluid to a full 'permeation', or, in other words, the water in the tissue had the same urea concentration as the external solution. Without cyanide, it entered to a much higher level. With 20 mg./100 ml. urea outside the mean increase in 37 min. over the inactive kidney was 17 mg./100 g. kidney, but the range was as high as a 47 mg./100 g. increase. In 37 min. therefore there was accumulated by the special activity of the kidney as much urea as was contained in a volume of external fluid 0.85 of the renal volume and the accumulation could reach more than double this figure. The accumulated urea diffuses as freely from the kidney as urea entering the cells passively as shown by a study of the diffusion curve and coefficients of diffusion. Could the urea accumulation be explained by the entrance of external fluid—produced by some pressure difference—at one region of the kidney, and then the urea be concentrated by water absorption at another region? Such an explanation has been advanced for similar accumulation of phenol red in the isolated kidneys of *Rana catesbiana* by Richards & Barnwell [1928]. These authors, however, did not consider either the total accumulation with respect to the water absorption this would entail or the pressure requirements. It may be noted, first, that from Krause's data as given by Pütter [1926] and calculating for a mean frog weight of 25 g. (as used in the experiments described above) an average of 55 mg. urine will be excreted by one kidney in 37 min., or 1.4 times the mean volume of the kidney. As considered above, the accumulation of urea would require 0.85 of the renal volume absorbed within 37 min., but since from the diffusion rate of accumulated urea from the kidney immersed in Ringer fluid (free from urea), 73 % will have been lost within this period, then upwards of  $4 \times 0.85$  the renal volume (or about twice the amount of urine normally passed within the period), will need to be absorbed into the isolated organ. If this amount of fluid were interpreted as coming along the blood vessels and through the glomeruli under the suction pressures produced somewhere down the tubule, then it may be held that the capsule of Bowman and glomerular capillaries would close like a valve, since from their histological structure they can offer no appreciable resistance to the pressure differences necessary. It would be similar to expecting a flow of fluid through an

immersed organ by suction on its vein, and the same would apply to fluid entering from the ureter. If again we were to suppose the fluid to have been sucked across the upper tubule walls, then even with large differences in osmotic pressure, produced by a considerable dilution of the external fluid, water itself does not enter isolated tissues with anything like the rapidity required. Thus, as shown by Buglia [1909] for the isolated gastrocnemius of the frog, an increase of only 9% of its weight occurs after 37 min. immersion in  $N/25$  saline. (The special excretion of urea appears to be confirmed by a study of inulin accumulation under conditions similar to the urea experiments and from a Ringer fluid containing 2% or more of inulin. Such experiments are in progress here, and so far they show that, compared with urea, inulin is not appreciably accumulated by the active kidney over that entering the inactive organ. Such inulin experiments do not in themselves outrule active excretion of inulin since a much shorter section of tubule may be involved.)

The difficulties against showing the same effect of urea accumulation in the mammalian kidney are considerable. First, only sections of the cortex can be used in order to secure an adequate oxygen supply, and in these the tubules will open freely into the external solution. Seeing that the diffusion of urea will take place from these openings at anything from 12 to 50 times as fast as through the cells, it is obvious that this will greatly lower the lumen concentration and the chances of demonstrating secretion. Also, even with thin sections, the tissue will scarcely be functioning at its normal level. We have, however (as indicated above), repeated such experiments with the kidneys of the cat and dog, and the results are rather curious. After perfusing the kidney with urea 0.2% and cyanide (securing good urine flow and thereby largely diminishing the urea in tubules if there remained any secretory activity) the distribution of the urea permeation gave 100 as the mode and a mean value of  $94\% \pm 2.1$ . When the sections were transferred after perfusion to a diffusion chamber containing the same fluid as that used in perfusing, the 'permeation' rose to 107%; with cyanide omitted from the diffusing fluid, it reached 109% (see Fig. 3). Here then it may be said that a definite increase over 100 occurred in the diffused sections, but that the effect is almost as great in the presence of cyanide which in the mammalian kidney, at least, leaves a very appreciable residual oxidation. The results, therefore, do not give the same clear-cut decision as with the frog's kidney.

*Urea 'permeations' in the central nervous system.* Whereas the urea

permeation of muscle or of renal cortex is at or close to the expected value of 100, that of the central nervous system of the rabbit and cat is definitely less, indicating that some region is quite impermeable to urea or that some proportion of the water is bound.

A curious result follows the injection of urea. A certain region of the nerve tissue is rapidly permeated, there being very little or no increase in the permeation value from 30 to 60 min., and the value of 30-40% is probably reached very quickly. From this region of the brain the urea diffuses with the same rate as from kidney or muscle sections, as evidenced by the diffusion coefficient. After this rapid entrance into about 30% of the tissue water there is a very slow diffusion into another region which is not complete even after  $4\frac{1}{2}$  hr.

It is clear, therefore, that the total volume of the tissue may be divided into four volumes or 'spaces' when we include the free intercellular space (inulin 'permeation') and the water into which the urea does not appear to enter even after an indefinite contact with the tissue.

The following explanation may be advanced as to what these tissue 'spaces' signify. The considerable difference in rate of entrance of urea may be explained by a rapid entry into nerve cells and a comparatively slow passage into the conducting fibres. This should be evident also for the spinal cord, but a smaller 'permeation' should be observed in the initial short period. The few observations made tend to support this view, since only a 15% 'permeation' was found with the cat's spinal cord after 25 min., whereas the cerebral cortex of the cat gave 44% 'permeation' in the same time, and the fullest 'permeations' for both tissues (corresponding to the normal values with respect to the blood urea) are 63.0 and 86.5% as judged from rabbit experiments. The question arises as to what is the significance of the 'space' indicated by the failure of urea to reach a 100% 'permeation' in nerve tissue. Since this 'space' is much greater for the cord than for the cerebral cortex, we may suppose it is connected with the conducting tissue and not with the nerve cells. Alternatively, it may not be a 'space' in any real sense but merely the effect of a solubility difference between urea in the myelin sheaths and in the blood.

Here account may be taken of the results of Riser, Valdiguie & Guiraud [1938], for the urea concentration of brain, muscle, blood, etc. after intravenous injection of urea into dogs (2-3 g./kg.). About 30 min. after the injection of urea 'permeation' in the brain would appear from their figures to be approximately 70% and that of muscle 90%. The data for the brain agree with ours (for the urethanized rabbit and cat) only

in so far as they show a considerably smaller value than for muscle, but differ much in the magnitude of the relative brain concentration. This is about twice what we have found. Our experiments were performed before those of Riser *et al.*, and since reading an account of their paper we have repeated and confirmed our results. Wherein lies the cause of the different effects described, whether it be due to different animal species or experimental conditions, does not at present appear.

*Diffusion coefficients.* It has been already shown that both for the frog's muscle and renal tissue the diffusion coefficient of urea is  $1.1 \times 10^{-5}$  (cm.<sup>2</sup>/min.) [Conway & Kane, 1934]. These values were obtained at a mean temperature of 18° C. From the usual effect of temperature on diffusion coefficients *in vitro* we could expect a value of about  $1.6 \times 10^{-5}$  at 38° C., but the mean value for the mammalian tissues at 38° C. is found to be  $8.0 \times 10^{-5}$ . As already indicated, however, the value depends largely on the thickness of the section (though for this most of the observations were made on renal tissue). Allowing for this effect the mean coefficient at a thickness of 0.5 mm. is  $2.5 \times 10^{-5}$  which is not far from the anticipated value. The coefficients for muscle and liver and cerebral cortex (first permeable region) appear to be of the same order as for the kidney, but for the cerebral cortex the effect of thickness of section does not seem to be of nearly the same consequence.

The diffusion coefficients of urea and of inulin through an agar gel at 38° C. are  $88.8 \times 10^{-5}$  and  $19.6 \times 10^{-5}$  or a ratio of 1.0 : 0.22. Through renal cortical tissue at 1 mm. thickness of section, the coefficients are  $8.30 \times 10^{-5}$  and  $3.36 \times 10^{-5}$  or a ratio of 1.0 : 0.38, the inulin coefficient being relatively higher. This is probably due to the greater relative effect for the inulin of the amount dissolved in extracellular water, since if we were to suppose only 8% of the external inulin diffusing freely as through water, it would account for such a difference.

#### SUMMARY

1. The inulin 'permeation' in rabbit muscle has a mean value of 8-9% indicating that this fraction of the total water (or 7% of the total muscle volume) is freely permeable to external inulin. This figure sets a maximum value for the intercellular spaces in the excised tissue. The value is much lower than the chloride 'permeation' which was found to be 15% under similar conditions.

2. The inulin 'permeation' of the cerebral cortex has a mean value of only 2%.

3. The inulin 'permeation' of the renal cortex contrasts markedly with that for muscle and brain tissue, being 41 % (mean value) for renal cortical sections immersed after sectioning in Krebs's fluid containing inulin, and of 56.6 % for freshly excised kidneys perfused with an inulin-Krebs fluid.

4. The mean urea 'permeation' in muscle was found to be 93 % (eleven observations).

5. The urea 'permeation' of renal cortical sections after perfusing with modified Krebs's fluid containing urea and cyanide was  $94 \pm 2.1$  (standard error of mean). When cortical sections of such perfused kidneys were immersed in modified Krebs's fluid containing urea and cyanide the value rose to  $107 \pm 3.2$ , and to  $109 \pm 2.8$ , when the cyanide was omitted.

6. The urea 'permeation' of the brain and cord of the rabbit was found to be 86.5 and 63.0 (two animals) respectively. On raising the blood urea to about six times its normal level by an intravenous injection, a urea permeation of 30-40 % is quickly reached with a subsequent slow increase, so that after 270 min. it stands at 62 %.

7. The mean diffusion coefficient of  $8.30 \times 10^{-5}$  (cm.<sup>2</sup>/min.) for urea has been found for all the tissues examined (thirty-eight observations) and of  $3.36 \times 10^{-5}$  for inulin in the kidney (seven observations).

The scatter of the figures is wide, but this is mainly due to varying thickness of section; for the diffusion coefficient without any very obvious reason increases with the section thickness, and when this is 0.5 mm.  $k$  is  $2.5 \times 10^{-5}$ .

The coefficient appears to be the same or similar for the different tissues; but the number of figures do not warrant any exact comparison.

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THE EFFECT OF HYPERTONIC GLUCOSE SOLUTIONS  
ON THE INFLOW OF NORMAL SALINE SOLUTION  
INTO THE SUBARACHNOID SPACE OF THE DOG

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ALTHOUGH the effect of hypertonic solutions on the cerebrospinal fluid pressure and brain volume of the cat has been the subject of careful study [Weed & McKibben, 1919*a*, *b*; Foley & Putnam, 1920; Foley, 1923], only scanty observations are available regarding the action of these solutions when administered to the dog. A study has been made in the following experiments of the effect of hypertonic solutions when administered intravenously on the rate of inflow of normal saline solution into the subarachnoid space of the dog. Special attention has been paid to the action of glucose solutions, although experiments were also performed with solutions of laevulose and of sodium chloride. The influence of these solutions on brain volume was also noted, and a few experiments were performed to determine their action on subarachnoid pressure.

*Experimental procedure*

The animals used in these experiments were by choice dogs varying from 7 to 10 kg. in weight and from 2 to 4 years of age. Owing to difficulties of supply from war conditions it was necessary, however, to make use of animals as they became available, and only limited selection was possible. The dogs were fed on the ordinary laboratory diet up to the night preceding the experiment when food was withdrawn; a liberal supply of water was always available. The experiments were performed in the early afternoon. Intratracheal ether administered by a pump was the anaesthetic throughout the experiments. Care was taken to maintain anaesthesia as even and as light as practicable. The solutions heated to body temperature were introduced from a burette fitted with a Mariotte tube into a tributary of the great saphenous vein in the region of the

ankle. A standard rate of inflow of 3 c.c./min. was maintained whenever possible. Nearly all the animals were able to tolerate this rate of inflow without displaying signs of circulatory disturbance. The cerebrospinal fluid pressure was measured in the usual way with a needle in the cisterna magna. The rate of inflow was determined at a constant pressure of 300 mm. normal saline solution throughout the experiments. Reference should be made to an earlier paper [Bedford, 1938] for further details of technique and for a description of the apparatus used to maintain the pressure and to measure the rate of inflow. Brain volume was determined by direct observation through a  $\frac{3}{8}$  in. trephine hole over the convexity of a cerebral hemisphere. This was done at the end of an experiment after the subarchnoid pressure had been set at its original level. Experiments were continued whenever possible for  $1\frac{1}{2}$  hr. after the solutions had been introduced.

#### THE EFFECT OF SOLUTIONS OF GLUCOSE

##### *Isotonic glucose solution*

Glucose A.R. was used throughout these experiments. The administration of glucose in a 5.4% concentration in distilled water in a proportion of 3 c.c./kg. body weight caused only a temporary diminution in inflow which disappeared as soon as the injection was completed, and inflow proceeded at the original rate throughout the rest of the experiment. Large volumes such as 30 c.c. or more per kg. produced a marked diminution in inflow, and the original rate had frequently not been attained at the end of  $1\frac{1}{2}$  hr. When the brains of these animals came to be examined at the end of the experiments they were generally found to be moderately swollen and to bulge slightly through the trephine hole; in no instance was a brain found to be shrunken.

##### *Hypertonic glucose solution*

##### (1) *The effect on inflow into the subarachnoid space*

Solutions of glucose in a 50 and a 25% concentration in distilled water were administered in a proportion of 3 c.c./kg. body weight. Eleven experiments were performed with a 50% and four with a 25% solution. The effect of a 50% concentration will be considered first. Considerable variation was observed in the response of individual animals. The introduction of the solution invariably caused an increased rate of inflow: this generally became evident after the injection had been in progress for 3 min., and the maximum rate was usually attained during the 3 min. which immediately preceded the cessation of the injection. The maximum



increase in inflow usually amounted to 50 % of the initial rate; occasionally inflow was doubled and in one instance trebled. Inflow began to decline immediately on terminating the injection; variations were observed in the time required for the attainment of the original rate. In eight experiments the initial rate had been resumed after 15 min., in two after 30 min. and in one the initial rate had not been attained after 1 hr. This

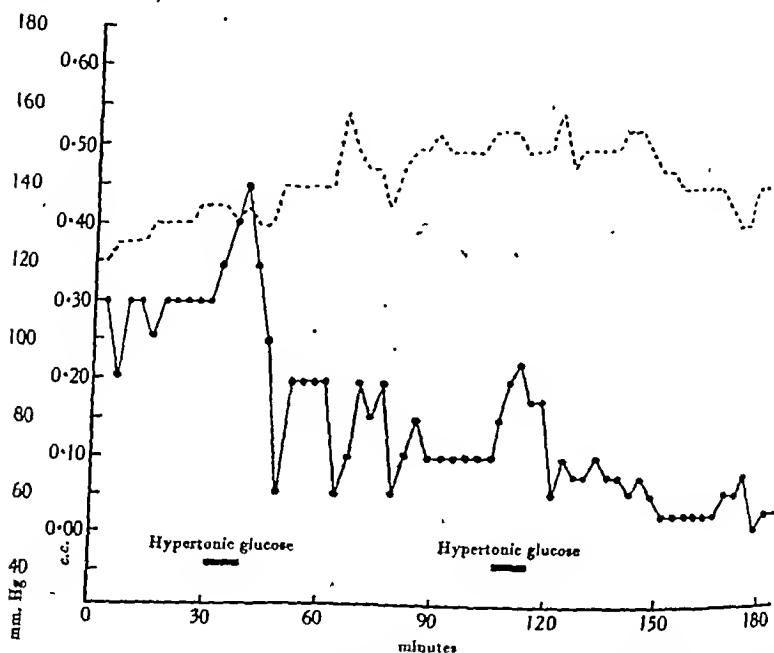


Fig. 1. The effect of hypertonic glucose solution (50%) on the rate of inflow of normal saline solution into the subarachnoid space. The rate of inflow was determined at a constant pressure of 300 mm. normal saline solution throughout the experiment and is expressed in terms of volume of solution entering the subarachnoid space during periods of 3 min. •—• Inflow of normal saline solution. - - - - Systolic pressure in femoral artery. Total volume of glucose solution administered at each injection 24 c.c. Weight of dog, 8 kg. The brain was found to be moderately swollen at the end of the experiment.

last finding may have been the result of faulty technique, for difficulty was encountered in this particular experiment in introducing the needle into the cisterna magna. No relationship was observed to exist between the extent of increase in inflow and the length of time required for return to the original rate. The original rate of inflow was not, however, maintained and the decline was progressive. After 1 hr. inflow averaged 50 % of the original rate in eight experiments, and in five of these which were

continued for another hour the reduced rate was maintained with evidence in most instances of further decline. A typical experiment is indicated in Fig. 1.

The effect of administering a second dose of glucose equivalent in volume and concentration to the first was now found to be less effective in increasing inflow, and the onset of reduced inflow was accelerated.

Two experiments were performed using a 50 % solution of glucose in normal saline solution; the findings were identical with those obtained when the glucose was administered in water.

After trephining the skull and opening the dura, the brain was found to bulge abnormally and to present clear evidence of oedema in about half the animals; in the other half the brain did not bulge abnormally, but signs of early oedema could generally be detected. In no instance was the brain found to be shrunken.

Little difference was observed in the effects produced by 50 and by 25 % solutions of glucose. The initial increase in inflow was somewhat smaller in the case of the 25 % solutions, although the time required for the onset of reduced inflow and the degree of reduction at the end of the experiments appeared to be identical.

## (2) *The effect on the pressure of the cerebrospinal fluid*

The effect of a 50 % solution of glucose on the pressure of the cerebrospinal fluid was studied in four animals. The quantity of solution introduced and the method of administration were the same as in previous experiments. A typical experiment is indicated in Fig. 2. The pressure generally began to fall after the injection had been in progress for 3 min.; the maximum fall observed in a single experiment was 35 mm. normal saline solution and the average maximum fall for the four experiments was 25 mm. normal saline solution. A gradual rise of pressure began immediately the injection was completed, and the original level had been reached after 18 min. in all four experiments. From now onwards the pressure began to rise and continued to do so during the remaining part of the experiments. The greatest rise in pressure observed in a single experiment was 80 mm. normal saline solution; in this instance the cerebrospinal fluid pressure was doubled. The smallest rise observed was 10 mm. normal saline solution, which was equivalent to an 8 % rise in pressure. At the end of two of the experiments the cerebrospinal fluid pressure was set at 300 mm. normal saline solution, and the rate of inflow into the subarachnoid measured for 15 min. Inflow was found to be proceeding in each instance at an average rate of 0.12 c.c. for a 3 min.

period. The average rate of inflow at 300 mm. normal saline solution in twelve control experiments was found to be 0.25 c.c. for a similar period.

An examination of the brain for oedema at the end of the experiments revealed a moderate degree of this condition in all four animals.

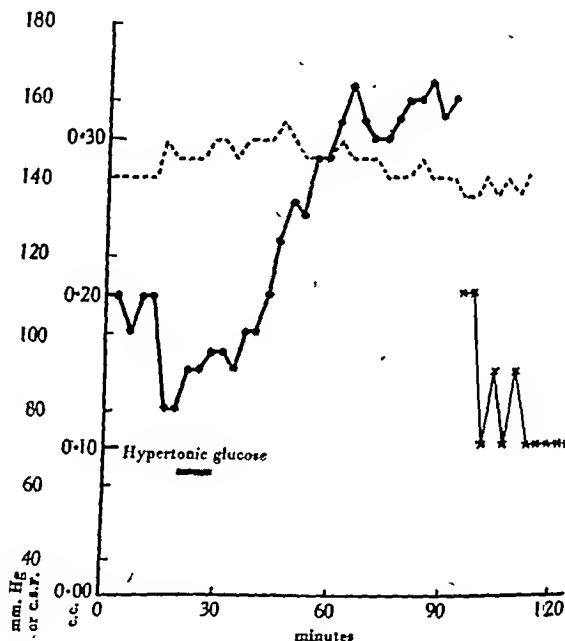


Fig. 2. The effect of hypertonic glucose solution (50%) on the pressure of the cerebrospinal fluid. After the experiment had been in progress for 90 min., the cerebrospinal fluid pressure was set and maintained at 300 mm. normal saline solution and the rate of inflow determined. ●—● Cerebrospinal fluid pressure. ×—× Inflow of normal saline solution. . . . . Systolic pressure in femoral artery. Constants as in Fig. 1. Total volume of glucose solution injected, 21 c.c. Weight of dog, 7 kg. The brain was found to bulge on opening the dura at the end of the experiment.

*The effect of hypertonic laevulose solution on the rate of inflow into the subarachnoid space and on the pressure of the cerebrospinal fluid*

A 50% solution of laevulose B.D.H. in water was used in these experiments. The volume of solution introduced and the method of administration were precisely the same as in the previous experiments. The effect on inflow was studied in three animals. Laevulose appeared

to have an action indistinguishable from that of glucose when administered in the same concentration. The effect on the pressure of the cerebrospinal fluid was studied in one animal only when it was found to be similar to that of glucose.

### *The effect of solutions of sodium chloride*

#### (1) *Normal sodium chloride*

The introduction of 3 c.c. normal sodium chloride solution (0.9% sodium chloride A.R.) per kg. body weight appeared to be without influence on the rate of inflow into the subarachnoid space. Large quantities of solution, ranging from 10 to 30 c.c./kg. caused a diminution in inflow which was roughly proportional in degree and duration to the volume introduced. Diminution in inflow was generally associated with moderate swelling of the brain; it was never observed in association with a shrunken brain.

#### (2) *Hypertonic sodium chloride solution*

Sodium chloride A.R. in a 20% concentration in water was administered to five animals. The volume of solution introduced and the method of administration were the same as in the previous experiments. Considerable variation was observed in the responses of different animals. It will be noticed in the experiment indicated in Fig. 3 that the injection caused at first a marked increase in the rate of inflow. The maximum rate of inflow was over four times the average rate prior to injection, and half an hour was required after the cessation of injection before the original rate was attained. Nevertheless, at the end of this experiment, inflow was proceeding at half the original rate. In two of the experiments the original rate was attained within 21 min. of the cessation of injection. On the other hand, 1 and 1½ hr. respectively were required in the two remaining experiments. In all experiments, however, the diminution in rate of inflow was continuous after the cessation of injection. When the brains came to be examined at the end of the experiments moderate oedema was present in three, while the other two appeared normal to naked eye inspection. It was observed as a general though not an invariable rule that the extent of reduction in inflow at the end of an experiment and the degree of cerebral oedema were in direct relationship. A marked reduction in inflow was nearly always accompanied by unmistakable evidence of oedema. A similar relationship was also observed

after the use of hypertonic glucose and laevulose solutions. No experiments were performed on the effect of hypertonic saline solution on cerebrospinal fluid pressure.

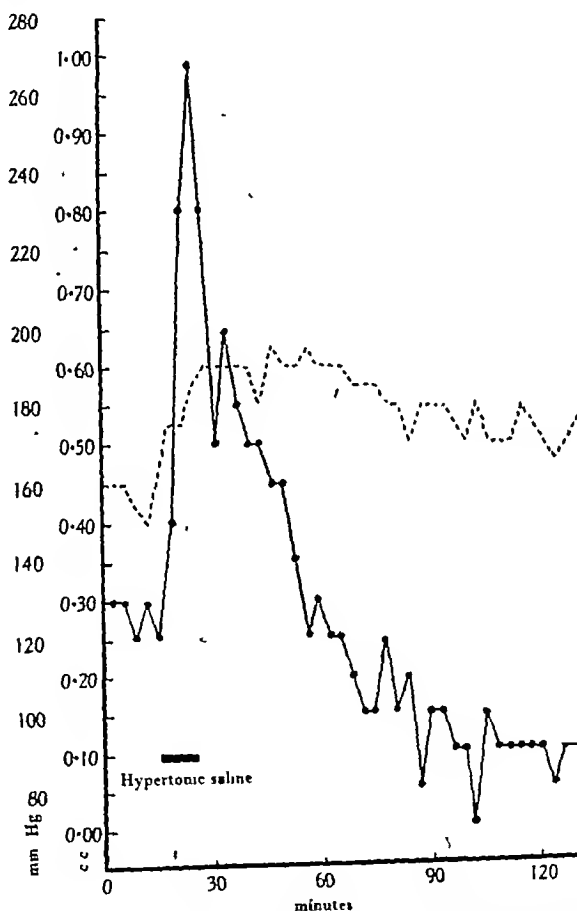


Fig. 3. The effect of hypertonic saline solution (20%) on the rate of inflow of normal saline solution into the subarachnoid space. Constants as in Fig. 1. Total volume of solution injected, 30 c.c. Weight of dog, 10 kg. The brain was found to be moderately swollen at the end of the experiment.

### DISCUSSION

It would appear from the above experiments that the intravenous administration of a 50% concentration of glucose in water in a proportion of 3 c.c./kg. body weight produces after a relatively short interval a steady diminution in the rate of inflow of normal saline solution into the sub-

arachnoid space, and the frequent finding of an oedematous brain at the end of the experiments suggests very strongly that the diminution in inflow of the saline is caused by an obstruction of the subarachnoid channels. It would seem therefore that the most characteristic effect of hypertonic glucose solution on the brain of the dog is the rapid production of cerebral oedema. It was thought that this early onset of oedema may have been in some way related to the introduction of relatively large quantities of normal saline solution immediately following the injection of hypertonic glucose solution. This, however, is rendered highly improbable by the experiments on the effect of hypertonic glucose on the pressure of the cerebrospinal fluid when no foreign solution was introduced into the subarachnoid space. In these experiments the onset of the rise in the pressure of the cerebrospinal fluid above its original level generally coincided with the beginning of diminished inflow in the other group of experiments.

It must be admitted that the experiments provide little evidence regarding the effects of hypertonic glucose solutions on the formation and the absorption of the cerebrospinal fluid; any effects that may have been produced are obscured by those brought about by changes in brain volume. It is interesting to observe that no difference was noticed between the actions of laevulose and of glucose.

The injection of sodium chloride in a 20 % concentration gave rise to a more prolonged period of increased inflow. This concentration of sodium chloride has, of course, a much greater tonicity than a 50 % solution of glucose; nevertheless, a reduced rate of inflow was present after  $1\frac{1}{2}$  hr. in all experiments. The experiments on the effect of the injection of large quantities of isotonic solutions confirm the findings of Weed & McKibben [1919] on the cat. The decrease in inflow in these experiments was probably caused by the brain, swollen in consequence of the increase in blood volume, obstructing the circulation of cerebrospinal fluid in the subarachnoid channels. Evidence has been provided elsewhere [Bedford, 1939, 1941] that an increase in brain volume resulting from a dilatation of its blood vessels may rapidly bring about an obstruction of the subarachnoid channels in the dog.

It will have been observed that a standard injection of 3 c.c. of solution per kg. body weight was used throughout the experiments. The solutions were administered in this proportion because it represents at least twice the quantity of 50 % glucose solution usually recommended for the relief of cerebral oedema in the human subject [Browder, 1930; Sachs, 1931; Brain, 1933].

Weed & McKibben [1919 *a, b*], working on cats, found that the characteristic effect of the intravenous administration of hypertonic solutions was a sustained fall in the pressure of the cerebrospinal fluid and a marked decrease in brain volume; no record is made by these workers of the occurrence of cerebral oedema in any of their experiments with hypertonic solutions. Foley & Putnam [1920], who appear to have worked mainly on cats, and Foley [1923], who used cats exclusively, obtained similar results when the hypertonic solutions were administered by the alimentary tract. These findings are contrary to those obtained in the experiments under consideration, and it is concluded that the brain of the dog probably differs from that of the cat in its response to the intravenous administration of hypertonic solutions.

#### SUMMARY

1. A study has been made of the effect of the intravenous administration of hypertonic glucose solutions (50 and 25 %), at a standard pressure, on the rate of inflow of normal saline solution into the subarachnoid space of the dog. Observations were also made on the effect on cerebrospinal fluid pressure and on brain volume.

2. It is concluded that the most characteristic effect of hypertonic glucose solution is the rapid production of cerebral oedema.

3. Identical results were obtained in a limited series of experiments with hypertonic laevulose solution (50 %).

4. Hypertonic sodium chloride solution (25 %) had a similar action although the onset of the cerebral oedema was not so rapid.

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## A METHOD OF CONDUCTING A BIOLOGICAL ASSAY ON A PREPARATION GIVING REPEATED GRADED RESPONSES ILLUSTRATED BY THE ESTIMATION OF HISTAMINE

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BIOLOGICAL assays on isolated preparations are generally based on the principle of 'bracketing' doses of the unknown with doses of the standard until their effects are matched. This method does not lend itself readily to statistical analysis. Many workers do indeed attempt to estimate the accuracy of their assays by performing preliminary experiments with solutions of known composition. Such preliminary experiments are, however, inefficient and also apt to be misleading, as conditions often do not approximate to those of a real assay. It is thus preferable to deduct the error of an assay from the data of the experiment itself.

Special difficulties arise if the object of the experiment is to test whether two solutions have the same activity. As the two solutions are tested repeatedly chance variations occur, and owing to the lack of criteria for dealing with these the result frequently becomes more dubious the more the experiment is prolonged. It is in this type of experiment involving the setting up and testing of a 'null hypothesis' [Fisher, 1937], that statistical methods are most useful, since they provide a definite answer, provided that the question is put in the right terms and the experiment designed on sound lines.

The object of this paper is to describe a method of conducting a biological assay on a single preparation in such a way that a valid null hypothesis may be set up and the accuracy of the result may be estimated from the data of the experiment itself. The method has been applied to the assay of histamine on the guinea-pig's gut. The design is based on a simple plan used in field experiments on adjacent plots [Fisher, 1938]. The statistical argument has been largely adapted from the work of



Gaddum [1933] and Bliss & Marks [1939 *a*; *b*]. The performance of the assay and its statistical analysis are discussed in detail, and it is hoped that readers who are not acquainted with statistical methods will have no difficulty in following the main argument and performing the test.

### METHODS

The experiments were done on preparations of isolated gut from guinea-pigs. Most assays were performed at 28–32° C. At this temperature no spontaneous contractions of the intestinal strip occurred.

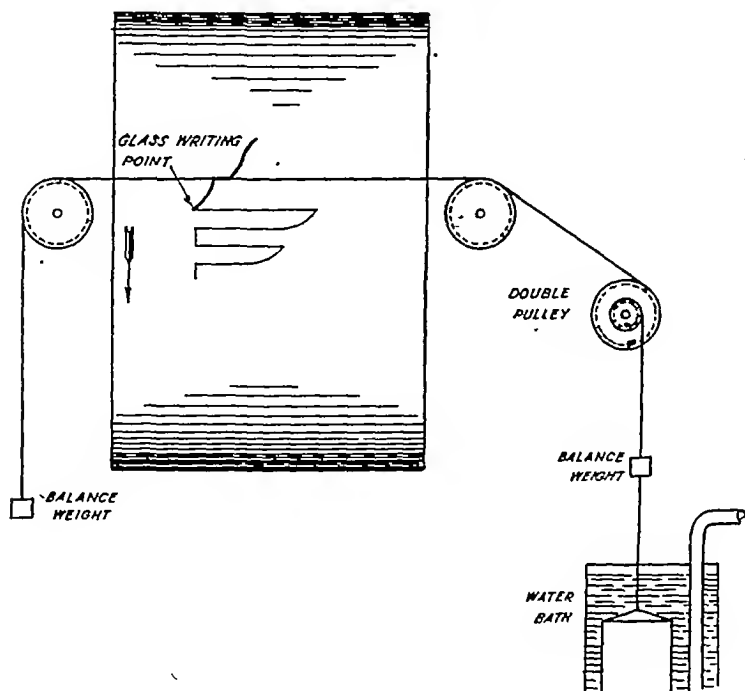


Fig. 1. Pulley system for linear recording of smooth muscle contraction.

A frontal writing lever was used in earlier experiments, but was discarded later owing to its relatively large error in recording at high angles of excursion. Instead, a pulley system of recording, shown in Fig. 1, was adopted; this provided a faithful record of the intestinal movements. A fine glass frontal writing point is attached to a horizontally moving silk thread which is kept taut by means of two small balance weights. Light vulcanite pulleys are used, and if magnification is desired it can be obtained by means of a double pulley as shown in Fig. 1. In the present

experiments, however, a single pulley without magnification was used in its place.

The bath volume was 25 c.c. and that of the test solutions added to the bath usually 1 c.c. Solutions were added at intervals of 3 min.

#### PERFORMANCE OF THE ASSAY

The assay is based on the assumption that, over the range of concentrations used, the contraction of the gut increases linearly with the logarithm of the dose.<sup>1</sup> It is carried out with the aid of only four doses, two of the standard and two of the unknown. They are chosen in a preliminary test and should fall within the limits of 10 and 90% of the maximum effect. The ratio of activity of the two doses of the unknown must be the same as that of the two doses of the standard, and the logarithm ( $d$ ) of this ratio should preferably be at least twice the logarithm ( $M$ ) of the ratio of activity of unknown and standard.

A suitable constant volume of test solution is added to the bath at regular intervals, the number and order of determinations being established at the outset according to the following scheme. The total number of determinations depends on the accuracy required, but it has to be a multiple of four, and every group of four consecutive determinations must contain each dose once. It is essential that within 'groups' doses should follow each other in random succession, which may be determined by means of random numbers or some physical process of randomization.

The logarithm of the ratio of potencies is given by

$$M = \frac{\bar{y}_u - \bar{y}_s}{b},$$

where  $\bar{y}_u - \bar{y}_s$  is the difference between the mean responses to unknown and standard, and  $b$  is the slope of the regression line plotted against log dose of standard.

If  $S(y_s)_1$  denotes the sum of all the effects (heights of recorded excursion) due to the larger dose of the standard and  $S(y_s)_2$ ,  $S(y_u)_1$ ,  $S(y_u)_2$  represent corresponding sums of effects of the smaller dose of the standard and larger and smaller dose of the unknown, and if  $N$  is the number of groups,

$$\bar{y}_u - \bar{y}_s = \frac{S(y_u)_1 + S(y_u)_2 - S(y_s)_1 - S(y_s)_2}{2N} \quad \text{and} \quad b = \frac{S(y_u)_1 + S(y_s)_1 - S(y_u)_2 - S(y_s)_2}{2\lambda d}.$$

Defining  $S(y_u)_1 + S(y_u)_2 - S(y_s)_1 - S(y_s)_2 = A$   
and  $S(y_u)_1 + S(y_s)_1 - S(y_u)_2 - S(y_s)_2 = B$

<sup>1</sup> Any other function of the dose giving an approximately linear relationship between dose and effect over a given range can be substituted for the logarithm without essential modification in the method of assay.

the expression for  $M$  becomes

$$M = \frac{A}{B} d.$$

*Graphical presentation.* Fig. 2 illustrates an experiment in which two known solutions of histamine—termed for convenience ‘standard’ and

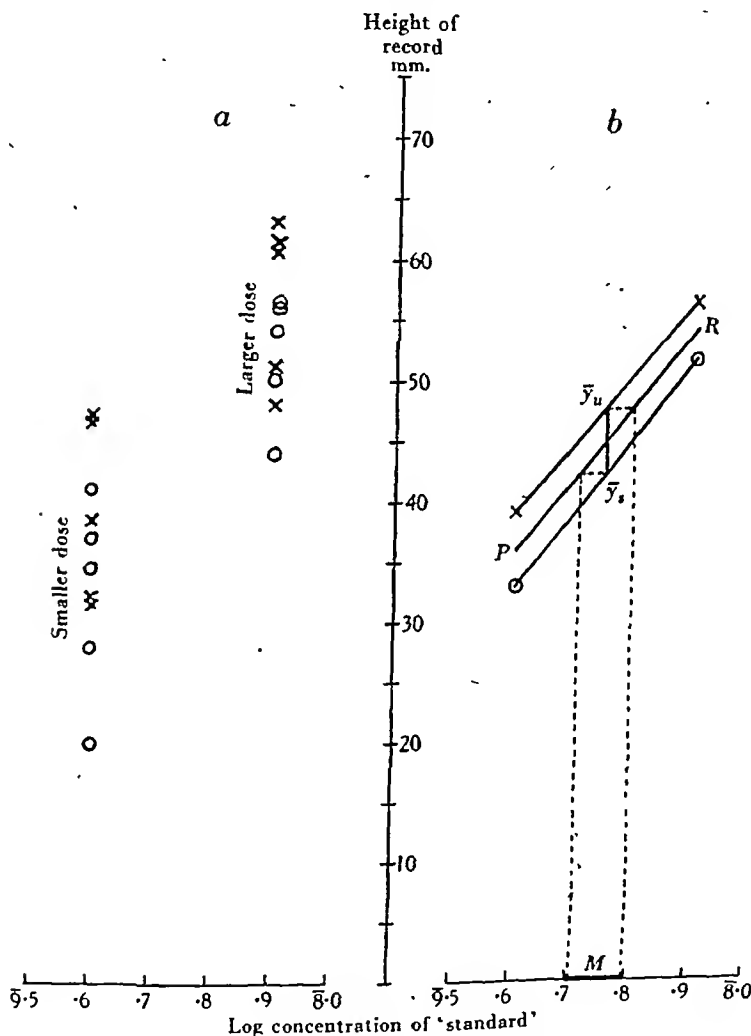


Fig. 2a. Effects of two doses of ‘standard’ (O) and two doses of ‘unknown’ (x). Ratio of activities 4:5;  $d=0.30103$ ;  $N=5$ .

Fig. 2b. Graphical determination of  $M$ .  $PR$  is the average regression.

'unknown'—were compared. The ratio of activity of the two solutions was 4:5, and the concentration ratio of doses was 1:2 ( $d=0.30103$ ). The effects are plotted against log concentration of standard.

Fig. 2*a* shows the scatter of results and the overlapping of effects due to standard and unknown. In Fig. 2*b* the mean effects of the four doses have been computed, and with their aid and the use of the mean regression line  $M$  is determined. The slope of  $PR$ , the mean regression line, is an average of the estimated slopes for standard and unknown.

Graphically  $M$  works out at about 0.09, this being the logarithm of 1.23, the estimated difference of activity is approximately 23%. The data of this experiment will be worked out in detail in the following sections.

#### OUTLINE OF THE STATISTICAL ANALYSIS

Fig. 3*a* illustrates the sequence of injections and the size of single responses in the above experiment, and Fig. 3*b* once more the mean effects of the four doses. The first object of the statistical analysis is to find out whether these mean effects differ from each other significantly, compared with the experimental error. In the simplest type of experiment the experimental error would be constituted by the variations in response to repeated tests with the same dose of histamine. In the present experiment, involving grouping, determination of the experimental error is somewhat more complex.

Fig. 3*a* shows that the mean 'group' response varies considerably in the course of the experiment, indicating marked changes in the sensitivity of the preparation. The effect of these variations in sensitivity has been largely eliminated from the experimental comparisons by the method of grouping which ensures that each dose is given at various levels of sensitivity and thus provides a well-balanced mean estimate for each dose. It is essential, however, that the differences between groups should be eliminated not only from the experimental comparison but also from the estimate of error, by the methods of the analysis of variance described in the following section. As a result, the estimate of the experimental error is reduced to the same value as if the mean sensitivity of groups had not changed in the course of the experiment. This is illustrated by Figs. 3*c* and 3*d*. The former shows the varying effects produced in the course of the assay by the same dose of histamine, the latter the same effects after eliminating the variations between groups. The adjusted effects are much more homogeneous and show a marked reduction of the variation ascribable to experimental error.

Having thus reduced the experimental error, the next step consists in extracting the maximum amount of useful information from con-

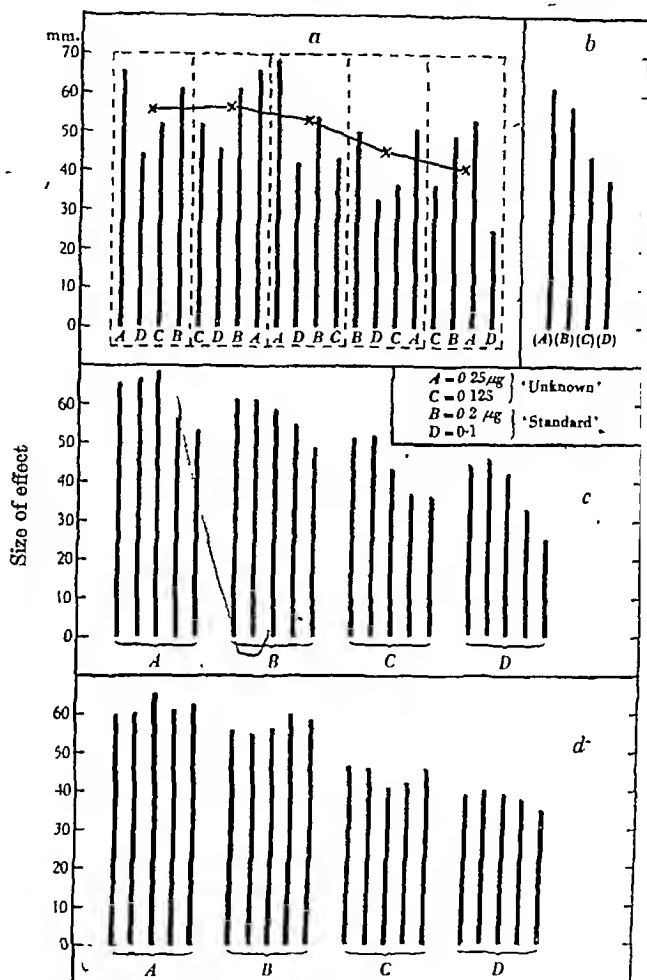


Fig. 3. Data from same experiment as in Fig. 2.

Fig. 3a. Order of tests and height of responses. Crosses indicate the mean response in successive groups.

Fig. 3b. Mean response to two doses of 'standard' and two doses of 'unknown'.

Fig. 3c. Successive responses to the same dose of histamine.

Fig. 3d. Values from Fig. 3b corrected to represent the effects that would have been obtained if the mean sensitivity of groups had not changed in the course of the assay.

trasting the mean effects of the four doses as presented in Fig. 2b. Three 'independent comparisons' can be made, and the significance

of each contrast may be assessed by relating it to the experimental error.

The effects of the two doses of the standard may be contrasted as a group with those of the two doses of the unknown. If variation between the two groups is significantly greater than the experimental error, it may be concluded without further assumptions that the two solutions differ in activity.

Secondly, the effects of the larger doses of both standard and unknown may be contrasted with those of the smaller doses. This is, in fact, a test for regression, since unless the larger dose produces a significantly greater effect than the smaller dose, no estimate of the regression coefficient and consequently no quantitative estimate of activity can be made.

Lastly, the sum of the effects of the larger dose of the standard and the smaller dose of the unknown may be contrasted with the sum of the other two effects. This test is a measure of parallelism, since if the two sums are equal the regression lines must be parallel. It cannot, of course, be expected, owing to chance variations, that the mean regression lines of standard and unknown should be perfectly parallel. If, however, the deviations from parallelism are significantly greater than the experimental error great caution must be used in the interpretation of results.

### *The analysis of variance*

The analysis of variance is 'a simple arithmetical procedure by means of which the results may be arranged and presented in a single compact table, which shows both the structure of the experiment and the relevant results in such a way as to facilitate the necessary tests of their significance' [Fisher, 1937]. It is essential, with the present method of assay, to compute an analysis of variance for each experiment, since it provides the error component for determining the limits of accuracy of the assay and leads to the various tests of significance outlined in the preceding section.

A typical analysis of variance computed from the data of the histamine assay previously quoted is shown in Table 2. The variate (Table 1) is the recorded maximum height of contraction produced by the addition of 1 c.c. of histamine solution to the bath. Table 2 shows that in the analysis five distinct sources of variation have been isolated. For each source of variation an expression called the *sum of squares* (of deviations from the mean) is computed, which divided by the appropriate *degrees of freedom* (*df*) yields a *mean square*. The ratio of two mean squares in conjunction with the degrees of freedom from which they are derived affords a test of significance.

TABLE 1. Effects of four doses of histamine applied in five successive randomized groups

Dose	Groups					Sum
	1	2	3	4	5	
0.25 $\mu\text{g.}$ ( $U_1$ )	131	132	136	112	106	617
0.2 $\mu\text{g.}$ ( $S_1$ )	122	122	118	110	98	570
0.125 $\mu\text{g.}$ ( $U_2$ )	103	104	87	74	73	441
0.1 $\mu\text{g.}$ ( $S_2$ )	89	92	84	66	50	381
Sum	445	450	425	362	327	2009

TABLE 2. Analysis of variance of histamine assay

Source of variation	Sum of squares	Degrees of freedom	Mean square
Between groups	2976.7	4	744.18*
Between 'standard' and 'unknown'	572.45	1	572.45*
Regression	6661.25	1	6661.25*
Deviation from parallelism	8.45	1	8.45
Error	330.1	12	27.51
Total	10548.95	19	

\* Highly significant.

The computations leading to the test of significance will be briefly described. The computational scheme is adapted from Snedecor (1938).

Given that  $S_1, S_2, \dots, S_N$  = each sum of items in a group of four responses (sum of each column of Table 1), and  $S = S_1 + S_2 + \dots + S_N$ , the following items are computed and then summarized as shown in Table 2:

(1) The correction term  $= C = S^2/4N = 2009^2/4(5) = 201804.05$ .

(2) The sum of the squares of all items  
 $= S(X^2) = 131^2 + \dots + 50^2 = 212353$ .

(3) The 'total' sum of squares  
 $= S(X^2) - C = 212353 - 201804.05 = 10548.95$ .

(4) The sum of squares for groups  
 $= (S_1^2 + S_2^2 + \dots + S_N^2)/4 - C = (445^2 + \dots + 327^2)/4 - 201804.05 = 2976.7$ ;

and the corresponding mean square by division by  $(N-1)$ , the corresponding degrees of freedom:  $2976.7/(5-1) = 744.175$ .

(5) The sum of squares for

(a) variation between standard and unknown  
 $= A^2/4N = (617 + 441 - 570 - 381)^2/4(5) = 572.45$ ;

(b) regression  
 $= B^2/4N = (617 + 570 - 441 - 381)^2/4(5) = 6661.25$ ;

(c) deviation from parallelism

$$= [S(y_u)_1 + S(y_s)_2 - S(y_u)_2 - S(y_s)_1]^2 / 4N$$

$$= (617 + 381 - 570 - 441)^2 / 4N = 8.45.$$

For each of these three sources of variation, only a single degree of freedom is available and their mean square is thus numerically equal to their sum of squares.

(6) The *sum of squares* for error

$$= \text{total} - (\text{groups} + \text{standard } v. \text{ unknown} + \text{regression} + \text{parallelism})$$

$$= 10548.95 - (2976.7 + 572.45 + 6661.25 + 8.45) = 330.1,$$

and the corresponding mean square by division by  $(3N - 3)$  the corresponding degrees of freedom:  $330.1 / (15 - 3) = 27.51$ .

A useful partial check of computations is afforded by the expression  $(S^2(y_u)_1 + S^2(y_u)_2 + S^2(y_s)_1 + S^2(y_s)_2) / N - C$ , which must be equal to the sum total of the three sums of squares with a single degree of freedom. Thus  $(617^2 + 570^2 + 441^2 + 381^2) / 5 - 201804.05$  must be equal to

$$572.45 + 6661.25 + 8.45.$$

In fact, both expressions add up to 7242.15.<sup>1</sup>

The test of significance is made by relating each mean square thus computed to the error mean square. The ratio  $F = \text{larger mean square} / \text{smaller mean square}$  is formed, and the simple value of  $F$  thus obtained is compared with a tabulated value of  $F$ . If the sample value exceeds the tabulated value for the 5% level of probability of  $F$  it is likely to occur less than once out of twenty times by chance and is said to be significant; similarly, if it exceeds the 1% level it is said to be highly significant. The numerical value of  $F$  depends not only on the required level of probability but also on the degrees of freedom from which the two mean

<sup>1</sup> If, in the course of the assay, a wrong dose is given by mistake, or some other accident occurs, the missing item ( $X$ ) can be supplied with the aid of a formula proposed by Allen and Wishart and Yates [quoted from Snedecor, 1938]. Adapted to the present purpose the formula is

$$X = \frac{4D + NG - S}{3N - 3},$$

where  $D$  = the sum of effects produced by the same dose as the missing effect,  $G$  = the sum of effects produced in the same group as missing effect, and  $N$  and  $S$  retain their previous significance. The value of  $X$  is entered in the table as the missing response, and the analysis of variance proceeds as usual with this one modification that the degrees of freedom for error are reduced by unity.

Thus assuming that in Table 1 item 87 from column 3, row 3 were missing,

$$X = \frac{4(441 - 87) + 5(425 - 87) - 2009}{3(5) - 3} = 91,$$

and the degrees of freedom for error are reduced to 11.



squares forming  $F$  are derived. Thus if it is desired to find the value of  $F$  derived from  $n_1$  and  $n_2$  degrees of freedom for a given level of probability, the appropriate table of  $F$  by Snedecor [1938] (or the corresponding table of  $e^{2x}$  by Fisher & Yates [1938]) is entered at the column headed  $df=n_1$ , and the required value is found at the row headed  $df=n_2$ .

The analysis of variance in Table 2 yields the following  $F$ -values:

$F$  for variation between standard and unknown  $= \frac{572.45}{27.51} = 20.81$  (the 1 % point of  $F$  for  $n_1=1df$  and  $n_2=12df$  is 9.33).

$F$  for regression  $= \frac{6661.25}{27.51} = 242.13$  (1 % point of  $F=9.33$ ).

$F$  for deviations from parallelism  $= \frac{27.51}{8.45} = 3.26$  (the 5 % point of  $F$  for  $n_1=12df$  and  $n_2=1df$  is 243.9).

$F$  for groups  $= \frac{744.18}{27.51} = 27.05$  (the 1 % point of  $F$  for  $n_1=4df$  and  $n_2=12df$  is 5.41).

It may be concluded that there is a highly significant difference in activity between standard and unknown, and a highly significant regression between the smaller and the larger dose, making a quantitative estimate of activity possible. Deviations from parallelism are very slight, in fact the corresponding mean square is smaller than the error mean square, though not significantly smaller. Lastly, the high  $F$  value for groups is a justification of the experimental design, showing as it does highly significant variations between groups.

#### *The limits of error of the estimate of $M$*

$s_M$ , the standard error of  $M = (\bar{y}_u - \bar{y}_s)/b$ , may be computed from the expression

$$s_M = 2\sigma d \sqrt{N \frac{\sqrt{(A^2 + B^2)}}{B^2}},$$

where  $\sigma$  is the square root of the error mean square in the analysis of variance, and the other terms retain their previous significance. The formula is derived on the assumption that  $\bar{y}_u - \bar{y}_s$  and  $b$  are uncorrelated.

The standard error of a quotient whose numerator and denominator are uncorrelated is

$$s_{x/y} = \frac{x}{y} \sqrt{\left(\frac{s_x^2}{x^2} + \frac{s_y^2}{y^2}\right)}.$$

If  $\bar{y}_u - \bar{y}_s = A/2N$ ,  $b = B/2Nd$ ,  $s_{(\bar{y}_u - \bar{y}_s)} = \sigma/\sqrt{N}$  and  $s_b = \sigma/\sqrt{N}d$ , the above expression for  $s_M$  is obtained. It is equivalent to that given by Bliss & Marks [1939b].

The  $P$  0.99 limits of error of the assay are constituted by  $M \pm s_M t$ .

The value of  $t$  in this expression is obtained from a table of  $t$  [Fisher, 1938] for the 1 % level of significance and  $(3N-3)$  degrees of freedom, the same number as for error in the analysis of variance.

In the numerical example

$$M = \frac{A}{B} d = \frac{107}{365} \times 0.30103 = 0.08825$$

$$\text{and } s_M = 2 \sqrt{(27.51) \times 0.30103 \times \sqrt{5} \times \frac{\sqrt{(107^2 + 365^2)}}{365^2}} = 0.02016.$$

Since the value of  $t$  for the 1% level and 12df is 3.055,

$$M - 3.055s_M = 0.02666 \quad \text{and} \quad M + 3.055s_M = 0.14984$$

constitute the  $P$  0.99 limits of error of the assay. Taking the antilogarithm of these numbers and multiplying by 100 the estimate of activity is 122.6%, and the limits of error are 106.3 and 141.2%. The true activity, 125%, is well within the computed limits of error.<sup>1</sup>

In interpreting the expression for  $s_M$  it might profitably be transformed to

$$s_M = \frac{\sigma}{\sqrt{Nb}} \sqrt{\left(\frac{M^2}{d^2} + 1\right)}.$$

In this expression the ratio  $\sigma/b$  is an absolute measure of the variability of the preparation. Provided it remains constant the standard error diminishes with the square root of  $N$ , the number of groups in the assay. There are thus two factors limiting the accuracy obtainable. One is the total number of responses that can be elicited, and the other is the constancy of the preparation. If towards the end of an experiment the variability of the preparation increases, any further prolonging of the assay may well increase rather than decrease  $s_M$ .

$s_M$  is reduced by any decrease in the value of the quotient  $M/d$ . In practice, provided that  $M/d$  is not greater than 0.5, any further reduction of the ratio will not markedly alter the value of  $s_M$ .

The following relationship exists between the limits of error for  $M$  as derived from  $s_M$ , and the variance ratio test (F test) assessing the significance of the difference between standard and unknown. When  $M = 0$  the result of the two tests is identical, when, however,  $M > 0$  the F test is more discriminating. This is due to the fact that the F test is not affected by variations in the slope of the regression line.

<sup>1</sup> Dr I. O. Irwin has pointed out to me that these limits of error are only approximate since the  $t$  distribution is not strictly applicable to  $s_M$  in view of the error in  $b$ . He suggests computing the exact fiducial limits from an expression equivalent in our notation to

$$\frac{ABd}{B^2 - R} \pm \frac{2\sigma d}{B^2 - R} \sqrt{[N(A^2 + B^2 - R)]},$$

where  $R = 4t^2\sigma^2N$ , and the other terms retain their previous significance. Derivations of similar expressions will be found in Bliss [1936] and Fieller [1940]. It will be seen that the formula differs from the previous one by the introduction of the term  $(R)$ . In our example the two methods yield almost identical results, the fiducial limits by the above formula working out at 106.0 and 142.7%, but more important differences may arise if the slope is not well determined.

squares forming  $F$  are derived. Thus if it is desired to find the value of  $F$  derived from  $n_1$  and  $n_2$  degrees of freedom for a given level of probability, the appropriate table of  $F$  by Snedecor [1938] (or the corresponding table of  $e^{2x}$  by Fisher & Yates [1938]) is entered at the column headed  $df=n_1$ , and the required value is found at the row headed  $df=n_2$ .

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$F$  for deviations from parallelism  $= \frac{27.51}{8.45} = 3.26$  (the 5% point of  $F$  for  $n_1=12df$  and  $n_2=1df$  is 243.9).

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It may be concluded that there is a highly significant difference in activity between standard and unknown, and a highly significant regression between the smaller and the larger dose, making a quantitative estimate of activity possible. Deviations from parallelism are very slight, in fact the corresponding mean square is smaller than the error mean square, though not significantly smaller. Lastly, the high  $F$  value for groups is a justification of the experimental design, showing as it does highly significant variations between groups.

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where  $\sigma$  is the square root of the error mean square in the analysis of variance, and the other terms retain their previous significance. The formula is derived on the assumption that  $\bar{y}_u - \bar{y}_s$  and  $b$  are uncorrelated.

The standard error of a quotient whose numerator and denominator are uncorrelated is

$$s_{x/y} = \frac{x}{y} \sqrt{\left(\frac{s_x^2}{x^2} + \frac{s_y^2}{y^2}\right)}.$$

If  $\bar{y}_u - \bar{y}_s = A/2N$ ,  $b = B/2Nd$ ,  $s_{\bar{y}_u - \bar{y}_s} = \sigma/\sqrt{N}$  and  $s_b = \sigma/\sqrt{N}d$ , the above expression for  $s_M$  is obtained. It is equivalent to that given by Bliss & Marks [1939b].

The  $P$  0.99 limits of error of the assay are constituted by  $M \pm s_M t$ .

The value of  $t$  in this expression is obtained from a table of  $t$  [Fisher, 1938] for the 1% level of significance and  $(3N-3)$  degrees of freedom, the same number as for error in the analysis of variance.

TABLE 3. Assays with histamine solutions of known composition

Exp. no.	Method* of recording	Temp. ° C.	Activity of 'unknown' in terms of 'standard' %	No. of groups (N)	Test of significance† (F) for			Estimated activity %	Limits of error‡ of estimate %	σ/b
					Difference in activity of two solutions	Deviations from parallelism of regression lines				
1	f.l.	28	200	8	H	O		186.3	102.5-213.6	0.05458
2	f.l.	28	200	4	H	H		205.9	179.4-236.3	0.02863
3	f.l.	35.5	150	8	H	O		143.8	133.2-155.1	0.01929
4	f.l.	30	125	8	H	O		128.2	120.8-145.5	0.02044
5	f.l.	30	125	5	H	O		122.6	106.3-141.2	0.04326
6	l.r.	32	150	5	H	O		145.5	131.8-160.7	0.02762
7§	l.r.	32	110	10	S	O		108.3	100.2-112.9	0.03992
8	l.r.	32	125	3	H	O		133.0	106.7-165.8	0.04152
9	l.r.	32	115	6	H	H		114.9	106.3-124.1	0.02738
10	l.r.	32	110	6	O	O		108.6		0.05268
11§	l.r.	32	120	3	H	O		117.3	109.1-126.1	0.01444

\* f.l. = frontal righting lever; l.r. = linear method of recording.

† H = highly significant,  $P > 0.99$ ; S = significant,  $P > 0.95$ ; O = not significant,  $P < 0.95$ .

‡ P 0.99 limits of error are indicated in all experiments except no. 7, where P 0.95 limits are given.

§ One missing value interpolated.

## ASSAYS WITH SOLUTIONS OF KNOWN COMPOSITION

The results of a series of assays with known concentrations of histamine are shown in Table 3. Every assay comprises various tests of significance as well as an estimate of potency and the  $P$  0.99 limits of error.

In every experiment the estimated potency was well within the computed  $P$  0.99 limits of error. These varied considerably from one assay to the other, ranging from  $-8.2$  and  $+8.8$  to  $-27.7$  and  $+32.8$  %. These differences in the error range are largely due to a change in the numerical value of the ratio  $\sigma/b$ , measuring variability (last col. of Table 3). In two extreme experiments differences in variability were such that fourteen tests on one preparation would have been needed to furnish the amount of information provided by a single test on the other preparation.

The  $F$  values testing the difference in activity between 'standard' and 'unknown' are highly significant in all assays where solutions differed by 15 % or more. When concentrations differed by only 10 % the results were less definite. In two such experiments (Exps. 7 and 10) the estimated differences of activity were 6.3 and 8.6 %. Statistical analysis, however, showed that differences as great or greater than those found in Exp. 7 would have occurred by chance nearly five out of one hundred times, and differences as great or greater than in Exp. 10 almost twenty out of one hundred times. The differences are thus in neither assay highly significant and barely significant only in Exp. 7. Possibly a highly significant result would have been obtained by further prolonging the assay.

*Deviations from parallelism and linearity*

The assay in its present form includes a test of deviation from parallelism but no test of deviation from linearity of regression. If such a test were desired it would be necessary to determine more than two points on each regression line.<sup>1</sup> The test for deviation from parallelism gives, however, an indirect indication of deviation from linearity, which is sufficiently stringent for the present purpose.

The test of departure from parallelism is related to the test for quadratic regression, indeed, the two tests are numerically equal when  $M = \frac{1}{2}d$ . It is thus cubic regression which is more likely to lead to error, and the test for parallelism would be inadequate if the regression line had a pronounced sigmoid shape.

In the present series of assays with solutions of known composition, tests of deviation from both parallelism and linearity may be made, the

<sup>1</sup> A general discussion of assays involving more than two points on the regression line is provided by Bliss & Marks [1939 a, b].

TABLE 3. Assays with histamino solutions of known composition

Exp. no.	Method* of recording	Temp. °C.	Activity of 'unknown' in terms of 'standard' %	No. of groups (N)	Test of significance† (F) for		Estimated activity %	Limits of error‡ of estimate %	σ/b
					Difference in activity of two solutions	Deviations from parallelism of regression lines			
1	f.l.	28	200	8	H	O	180.3	162.5-213.0	0.05458
2	f.l.	28	200	4	H	H	205.9	179.4-236.3	0.02803
3	f.l.	35.5	150	8	H	O	143.8	133.2-155.1	0.01929
4	f.l.	30	125	8	H	O	128.2	120.8-145.5	0.02044
5	f.l.	30	125	5	H	O	122.0	106.3-141.2	0.04325
6	l.r.	33	150	5	H	O	145.5	131.8-160.7	0.02762
7§	l.r.	32	110	10	S	O	106.3	100.2-112.0	0.03992
8	l.r.	32	125	3	H	O	133.0	100.7-165.8	0.04152
9	l.r.	32	115	6	H	H	114.9	106.3-124.1	0.02738
10	l.r.	32	110	6	O	O	108.6		0.05208
11§	l.r.	32	120	3	H	O	117.3	109.1-120.1	0.01444

\* f.l. = frontal righting lever; l.r. = linear method of recording.

† H = highly significant,  $P > 0.99$ ; S = significant,  $P > 0.95$ ; O = not significant,  $P < 0.95$ .

‡ P 0.99 limits of error are indicated in all experiments except no. 7, where P 0.95 limits are given.

§ One missing value interpolated.

latter by treating the two doses of 'standard' and 'unknown' as forming a sequence of four different concentrations of the same solution. Such tests are presented in Table 4. The test of departure from linear regression was done according to standard methods [Snedecor, 1938, p. 317].

TABLE 4. Deviations from parallelism and linearity

Exp. no.	<i>F</i> for deviations from parallelism	<i>F</i> for deviations from linearity	5% level of <i>F</i>	1% level of <i>F</i>
1	0.03	1.55	4.32	—
2	30.85	31.40	5.99	13.74
3	1.45	3.81	4.32	—
4	1.06	1.58	5.12	—
5	0.31	0.77	4.75	—
6	0.05	0.67	4.75	—
7	0.29	1.69	4.22	—
8	3.81	4.76	5.99	—
9	9.23	9.26	4.54	8.68
10	0.36	0.71	4.54	—
11	0.95	2.30	6.61	—

Only in two experiments of the series (Exps. 2 and 9) deviations from parallelism occurred, and in both instances they were associated with significant deviations from linearity. In the rest of the assays there were no significant deviations from either. Thus in all experiments except two there was no reason to assume that the regression lines differed from linearity within the range of the experiment. Possible reasons for departure from linearity were in one experiment the use of a frontal writing lever at a high angle of excursion, and in the other the application of a dose producing an effect greater than 90 % of the maximum.

Tentative estimates of potency in these experiments, treating data as if regression were linear, gave results which were surprisingly close to the true value. This suggests that the assay is relatively insensitive to deviations from parallelism.

Besides non-linearity of regression various other factors may cause deviation from parallelism, such as differential deterioration of histamine during the period of assay,<sup>1</sup> failure to dilute standard and unknown equally in preparing the second dose of each and possibly qualitative differences between standard and unknown.

#### *The effect of grouping*

An indication of the importance of eliminating gradual changes in sensitivity taking place in the course of the assay is provided by the *F* value for groups as computed in the analysis of variance. Table 5 shows

<sup>1</sup> This is probably due to bacterial action and may be prevented by boiling up solutions briefly.

TABLE 5. Significance of variations between groups

Exp. no.	$F = \frac{\text{M.sq. for groups}}{\text{M.sq. for error}}$	Degrees of freedom		5% level of $F$	1% level of $F$
		$n_1$	$n_2$		
1	16.03	7	21	2.49	3.65
2*	4.28	3	6	4.76	9.78
3	2.37	7	21	2.49	3.65
4	10.40	3	9	3.86	6.99
5	27.05	4	12	3.26	5.41
6	2.06	4	12	3.26	5.41
7	2.40	9	26	2.27	3.17
8	12.69	2	6	5.14	10.92
9	2.94	5	15	2.90	4.56
10	1.25	5	15	2.90	4.56
11†	34.17	2	5	5.79	13.27

\* Latin square arrangement.

† A somewhat longer time interval occurred between the first and second group.

that in the majority of experiments this value is significant, proving that the variations in sensitivity are real. The direction of these changes is illustrated in Fig. 4, which comprises all the significant results. Perhaps the most common feature is an initial rise in sensitivity followed by a gradual decrease.

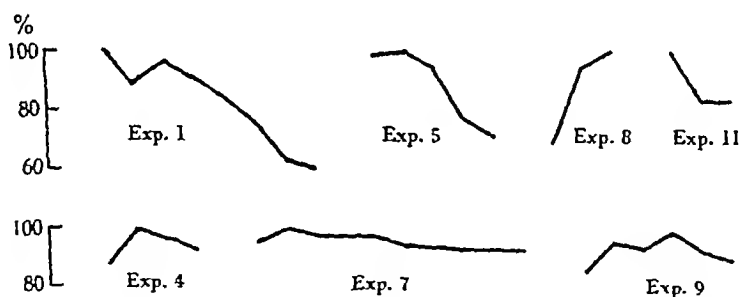


Fig. 4. Mean response in successive 'groups' in terms of maximum response.

In spite of irregularities a slight linear trend is usually discernible, and it is possible that a further reduction of the experimental error could be effected by other restrictions in design or the analysis of covariance. In one experiment (Exp. 2) in which a Latin square arrangement was used in order to equalize the order of tests within groups a substantial mean square was segregated for 'order of injections'. When, however, two further  $4 \times 4$  squares were appended in the same experiment (this part of the assay has been omitted in the text since some errors occurred) the mean square for order of injections became less than the error mean square.

### Independence of $\bar{y}_u - \bar{y}_s$ and $b$

In deriving the expression for  $s_M$ , the standard error of the ratio  $(\bar{y}_u - \bar{y}_s)/b = M$ , it was assumed that numerator and denominator of the fraction are independent. This assumption holds only if the slope of the



regression line does not alter in the course of the assay, since any real variation, as distinct from sampling variation, of the slope entails corresponding variations of the differences between effects. If the slope varied there should thus be significant correlation between mean difference of effects and slope in successive groups, or between successive values of  $(y_u)_1 + (y_u)_2 - (y_s)_1 - (y_s)_2$  and  $(y_u)_1 + (y_s)_1 - (y_u)_2 - (y_s)_2$ .

The strength of this correlation has been measured in each assay. In no instance did the correlation coefficient attain the 5% level of significance. When the correlation coefficients from all assays (except Exps. 2, 4 and 11) were pooled by means of the  $z$  transformation (Fisher, 1938) for 31 degrees of freedom, a non-significant negative correlation of  $r = -0.048$  was obtained. On the available evidence there is thus no reason to assume that  $\bar{y}_u - \bar{y}_s$  and  $b$  are correlated or that a change of slope of the regression line occurs in the course of the assay.

### SUMMARY

1. A method is described for conducting a biological assay on an isolated preparation in such a way that a null hypothesis may be adequately tested and the error of the assay may be estimated from the data of the experiment itself.

2. The method is applicable in its present form, if there is a linear relationship between log dose and effect over a given range, and if the slope of the regression line does not alter in the course of the experiment.

3. The method has been applied to the assay of histamine on the guinea-pig's intestine. In assays with solutions of known composition the  $P$  0.99 limits of error ranged in different experiments from  $-8.2$  and  $+8.8\%$  to  $-27.7$  and  $+32.8\%$ .

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## THE EFFECT OF SODIUM AND CALCIUM ON THE TOXICITY OF POTASSIUM IN MICE

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TRUSZKOWSKY & DUSZYNSKA [1940] have described the protective effect of desoxycorticosterone acetate against potassium chloride injected intraperitoneally in immature albino mice. In an attempt to repeat their work, we thought it advisable to investigate also the influence of sodium and calcium on potassium tolerance. The beneficial effects of a high intake of sodium chloride in Addison's disease, and in adrenalectomized mammals, suggested that sodium might afford some protection against potassium poisoning, in particular since the work of Zwemer & Truszkowsky [1936], Truszkowsky & Zwemer [1936, 1938] and of Marenzi [1938] tends to show that the symptoms of adrenocortical insufficiency may be due to an excess of potassium. Calcium might be expected to have some influence on the toxicity of potassium *in vivo*, since it appears to be protective against the action of excess potassium on the isolated mammalian heart.

### RESULTS

#### *Toxicity of potassium chloride*

Young male albino mice from the Institute's Farm colony were used in all tests, at a body weight of from 13 to 23 g. Preliminary trials showed that the steepest dose/response curve was obtained when, following Truszkowsky & Duszyńska [1940], potassium chloride in aqueous solution was given intraperitoneally in direct proportion to body weight. This was supported by the finding that, when all mice in a group were given the same dose of potassium, irrespective of their body weight, those killed were significantly lighter than the survivors (average weight of dead = 14.583 g., of living = 15.577 g., diff. = 0.994 g.,  $\sigma_{\text{diff.}}$  = 0.329 g., no. of mice = 100), whereas when the dose of potassium was in proportion to the weight of the mouse, such a difference was never found.

TABLE 1. Dose/response data for the toxicity of intraperitoneal KCl in young male albino mice, twenty-five mice per group. All tests were made on the same day.

Volume of injections = 0.2 ml./10 g. body weight.

Dose in mg./10 g. body weight	5	6	7	8
% mortality	0	32	76	96

Table 1 gives data from which a regression line for the toxicity of potassium chloride may be calculated; it is sufficient for our immediate purpose to show that the slope of the line is steep (*ca.* 18) and that the test is thus a sensitive one. The median lethal dose is in this case 6.37 mg. of potassium chloride per 10 g. mouse.

### *Effect of sodium chloride*

Sodium chloride in doses of 10 and 20 mg./10 g. body weight, given together with the potassium chloride in one injection, proved very effective in protecting the mice from the toxic effects of potassium

TABLE 2. The protective effect of NaCl against KCl poisoning, twenty mice per group, intraperitoneal injections.

Date of test	mg. KCl/ 10 g. body weight	Conc. of KCl %	mg. NaCl/ 10 g. body weight	Conc. of NaCl %	% mortality
14. viii. 41	6.6	3.3	—	—	0
	9.0	4.5	—	—	85
	9.0	4.5	20.0	10.0	0
15. viii. 41	9.0	4.5	—	—	90
	9.0	4.5	10.0	5.0	40
21. viii. 41	6.4	3.2	—	—	5
	6.4	1.6	—	—	5
	8.0	4.0	—	—	95
	8.0	4.0	10.0	5.0	10
	8.0	2.0	10.0	2.5	10

(Table 2). As the tonicity of the solutions was high, the effect of altering the volume and thus the concentration of the injections was investigated, and shown to be nil.

### *Effect of calcium chloride and of glucose*

Further simultaneous tests were next made, to determine the effect of calcium chloride given intraperitoneally with potassium chloride, and of glucose solution of high tonicity (approx.  $\equiv$  5% NaCl or 10%  $\text{CaCl}_2$ ) given with the potassium. The results, shown in Table 3, demonstrate the ineffectiveness of both substances, and the ineffectiveness of a merely hypertonic solution.

TABLE 3. The lack of effect of  $\text{CaCl}_2$  and glucose on the toxicity of KCl injected intraperitoneally, twenty mice per group. All tests were made on the same day.

mg. KCl/ 10 g. body weight	Conc. of KCl %	mg. $\text{CaCl}_2$ / 10 g. body weight	Conc. of $\text{CaCl}_2$ %	mg. glucose/ 10 g. body weight	Conc. of glucose %	% mortality
6.4	3.2	—	—	—	—	40
8.0	4.0	—	—	—	—	95
8.0	4.0	2.0	1.0	—	—	90
8.0	2.0	16.0	4.0	—	—	90
8.0	4.0	—	—	60.0	30.0	85

*Intravenous injections*

Potassium chloride is more toxic when injected intravenously in mice than when given intraperitoneally, but the response is very much dependent on the rate of injection. We therefore tried to inject at a constant rate, giving the total dose in about 10 sec. Data showing the effects of sodium and calcium chlorides on the toxicity of potassium chloride when injected with it are shown in Table 4.

TABLE 4. Effect of NaCl and  $\text{CaCl}_2$  on the toxicity of KCl when given intravenously in mice.

Date of test	mg. KCl/ 10 g. body weight (1% solution)	mg. NaCl/ 10 g. body weight (2% solution)	mg. $\text{CaCl}_2$ / 10 g. body weight	Conc. of $\text{CaCl}_2$ %	No. of mice	% mortality
2. x. 41	1.5*	—	—	—	12	100
	1.2	—	—	—	10	90
	1.0	—	—	—	10	40
	0.8	—	—	—	10	70
	1.2	2.4	—	—	10	10
	1.2	—	2.4	2.0	10	100
3. x. 41	—	—	1.0	2.0	5	0
	—	—	2.0	2.0	5	20
	—	—	4.0	2.0	5	60
9. x. 41	1.0	—	—	—	20	80
	1.0	—	0.5	0.5	10	80
	1.0	—	1.0	1.0	20	50
	1.0	—	1.5	1.5	10	60
	1.0	2.0	—	—	10	30

\* 1.5% solution.

It will be seen that sodium is again effective in lowering the toxicity of potassium, and that calcium may possibly have some slight effect in a dose of 1 mg./10 g. body weight, although it is itself toxic in relatively low doses. It seems, however, safest to conclude that these tests by intravenous injection substantiate the results obtained by intraperitoneal injection.

*Effect of desoxycorticosterone acetate*

Although this communication has been written primarily to record the effect of sodium and calcium, it would be misleading to omit mention of the fact that we have not been able to demonstrate any effect with desoxycorticosterone acetate. Thus the work of Truszkowsky & Duszyńska, which stimulated the investigation, is not substantiated by our own results. The Polish workers found that 1 mg. of desoxycorticosterone acetate significantly inhibited the response to potassium, but neither 1 nor 2 mg., administered as they gave it and by several other methods,

TABLE 5. The ineffectiveness of desoxycorticosterone acetate on the toxicity of KCl given intraperitoneally to mice. The KCl was injected in a volume of 0.2 ml. water, twenty mice per group.

Date of test	mg. KCl/ 10 g. body weight	Desoxycorticosterone acetate			Solvent	% mortality
		1st dose (mg.) (sub- cutaneous, 24 hr., prior to KCl)	2nd dose (mg.) (intra- peritoneal)	Period before KCl injection hr.		
12. ix. 41	8.0	—	—	—	—	90
	6.4	—	—	—	—	45
	5.4	—	—	—	—	5
	8.0	1.0	—	—	Arachis oil	95
	8.0	0.5	0.5	2.5	"	80
	8.0	—	1.0	3.0	"	90
19. ix. 41	10.0	—	—	—	—	100
	8.0	—	—	—	—	40
	8.0	2.0	—	—	Arachis oil	30
	8.0	1.0	1.0	3.5	"	70
	8.0	—	2.0	3.5	"	80
25. ix. 41	8.0	—	—	—	—	85
	6.4	—	—	—	—	50
	5.4	—	—	—	—	10
	8.0	0.5	0.5	3.5	Arachis oil	95
	8.0	0.5	0.5	3.5	Olive oil	90

had any inhibitory influence on the toxicity of potassium in our own mice (Table 5). Indeed, in the tests on 19 September 1941, there was evidence that it actually increased the toxicity, but this was probably due to the very low response to 8 mg. of potassium chloride in the controls.

## DISCUSSION

A mouse which has been injected intraperitoneally with a lethal dose of potassium chloride dies in a very characteristic manner. For from 5 to 10 min. it shows no symptoms. It then becomes very active, flicking its tail violently and leaping several times into the air, passes into convulsions, and dies with evident respiratory distress. The heart mean-

while continues to beat. Following an intravenous dose of potassium chloride, the mouse may collapse and die almost instantaneously, particularly if the injection is made rapidly, but it exhibits the above series of symptoms when the injection is given more slowly.

It does not seem, therefore, that death is due to heart failure, for the symptoms exhibited appear referable to the effects of potassium on the nervous system. This makes the ineffectiveness of the simultaneous administration of calcium more understandable, although it leaves the action of sodium unexplained. The life-saving action of sodium would seem to fall in line with its effects in adrenocortical deficiency, and perhaps even in shock, since it appears likely that some of the symptoms exhibited in these conditions may be due to a rise in blood potassium. The effectiveness of desoxycorticosterone in the two states mentioned is, however, greater than that of sodium, and it is disturbing that we have obtained negative results with it.

#### SUMMARY

1. The toxicity of potassium chloride when given either intraperitoneally or intravenously to male albino mice was decreased by the simultaneous administration of sodium chloride, but not of calcium chloride, glucose, or desoxycorticosterone acetate.

2. It is suggested that under the conditions of the tests, potassium kills by an action on the nervous system. The life-saving action of sodium chloride is in line with its beneficial effects in adrenocortical deficiency and in shock, states in which the blood potassium is higher than normal.

Our best thanks are due to Dr F. C. MacIntosh for advice and suggestions, and to Messrs Ciba Ltd., for the supply of desoxycorticosterone acetate.

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## THE EFFECTS OF DRUGS, SUGARS AND ALLIED SUBSTANCES ON THE ISOLATED SMALL INTESTINE OF THE RABBIT

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THE experiments described in this paper were carried out to analyse the stimulating action of sugars and related substances on the isolated intestine of the rabbit. In the course of the experiments the effects of acetylcholine, muscarine, eserine, phloridzin and atropine on the longitudinal and circular muscle layers of the intestine were examined in the presence and absence of glucose. Previous studies have usually been concerned only with the effect on the longitudinal muscle in the presence of glucose.

Rona & Neukirch in 1912 described the stimulating action of various sugars and related substances on the longitudinal muscle of the isolated intestine of the rabbit suspended in glucose-free Tyrode solution. Under these conditions the activity of the muscle diminished and disappeared. The subsequent addition to the solution of glucose, mannose or pyruvate revived and strongly stimulated the muscle. A weak action was obtained with galactose and lactate, whereas disaccharides were ineffective. Since the sugars disappeared from a solution into which a loop of intestine had been placed the stimulating effect was attributed to the ability of the substances to supply, in varying degree, the chemical energy necessary for the metabolism of the working muscle. The rhythmic activity and tone of the longitudinal muscle were regarded as being inherent properties of the muscle fibre. This concept does not become invalid if it is assumed that choline and acetylcholine which are continuously given off from the surviving intestine [Weiland, 1912; Le Heux, 1919; Feldberg & Rosenfeld, 1933; Donomae, 1934; Feldberg & Kwiatkowski, 1934] are the stimuli for augmenting the activity and tone of the muscle. In the absence of glucose the longitudinal muscle may become inexcitable to



the liberated choline and acetylcholine, normal excitability to them being restored by the addition of glucose or similarly acting substances. This explanation still attributes their stimulating action to an effect on the muscle fibre.

The sugars may, however, act by influencing the production and consequently the release of choline and acetylcholine. Magnus [1930] suggested that the stimulating action of pyruvate might be due to the formation of pyruvyl choline which was found to be more active than choline. He did not explain the stimulating action of the various sugars. The recent findings that these as well as pyruvate and lactate accelerate synthesis of acetylcholine in brain tissue in vitro [Mann, Tennenbaum & Quastel, 1938] and in a stimulated ganglion [Kahlson & MacIntosh, 1939] suggest such a mechanism as a possible explanation. Synthesis of acetylcholine in intestinal tissue has been demonstrated by Dikshit [1938] and, according to him, is mainly a function of the nerve plexus in the intestinal wall.

Our experiments were carried out in an attempt to evaluate the relative importance of the different mechanisms suggested above. According to Dikshit an intestinal preparation kept in the cold for a few days loses the property of synthesizing acetylcholine. A stimulating effect of glucose on such a preparation would therefore exclude the possibility of the stimulation being due to increased synthesis of acetylcholine. A negative result would be inconclusive, since the cold might prevent stimulation in any of several ways. In a second series of experiments the effect of glucose on the output of acetylcholine from the perfused intestine was examined. It might be argued that an increase in synthesis might not necessarily lead to an increase in output of acetylcholine. However, available evidence suggests that increased synthesis would stimulate the muscle only if acetylcholine were also liberated in increased amounts. A comparison of the stimulating action of glucose with that of acetylcholine or eserine in the absence of glucose should give further information on the problem.

There is at present no evidence that the stimulation of the intestine by various sugars and related substances is in each case due to the same mechanism and that they all act by 'replacing' glucose. If that were true stimulation should not occur when the substances are tested on preparations suspended in Tyrode solution containing glucose.

## METHODS

Rabbits weighing 1.5-2.5 kg. were killed by a blow on the back of the neck. The abdomen was opened in the middle line and the procedure then adopted varied according to the experiment.

*Suspension of an isolated piece of intestine.* A piece of 7-12 cm. from the duodenum or the upper third of the small intestine was used. When the response of the longitudinal muscle alone was examined it was suspended in such a way as to leave both ends open, the oral end being attached to a thread leading to a Lovatt Evans frontal writing lever. For studying the effect on the circular muscle layer a volume record was taken according to Trendelenburg [1917]. The caecal end was tied over a glass cannula connected by rubber tubing to the lower end of a 1 l. aspirator half-filled with the same solution as used for the bath. The upper end, attached by thread to the lever, was kept open for 30-60 min., during which time the inside of the gut was frequently washed out from the aspirator. The upper end was then closed by a ligature and the pressure inside the gut brought to  $2\frac{1}{2}$ -3 cm. water by raising the bottle. The upper opening of the aspirator was connected by rubber tubing to a small Krogh volume recorder. In the figures the upper tracing is the record of the intestinal volume, a diminution of which causes an upward stroke. The lower tracing is made by the suspension lever, an upwards stroke indicating a shortening of the gut. Some caution is necessary in the interpretation of the changes in volume. Shortening of the preparation may lead to some reduction in volume, but any reduction in volume which occurs without shortening must be due to a contraction of the circular muscle. The simultaneous record from the longitudinal muscle will avoid misinterpretation from this source. A strong but localized constriction of the circular muscle will have little influence on the volume, and reductions of equal extent may be brought about either by a strong contraction of part of the circular layer or by weak contraction of the whole wall. Therefore the effects on the circular muscle layer were further observed through the glass wall of the tank and the inside vessel. Tyrode solution, with and without glucose, was used, air bubbled through it and the temperature kept between 32 and 37° C. The volume of the bath was 35, 45 or 60 c.c. When no volume record was taken a 14 c.c. bath was often used. It was emptied by overflow and the substances were added with a syringe.

On cooled preparations the reactions on the longitudinal muscle only were examined. Pieces of the washed intestine were kept in glucose-free

Tyrode solution (at  $-1^{\circ}\text{C.}$ ) for 4–80 hr. Before use they were placed for 30–60 min. in glucose-free Tyrode solution at room temperature. The lumen was always full of secreted contents which were washed out and if necessary pressed out with a glass rod, before the preparations were suspended in the 14 c.c. bath.

*Perfusion of the isolated intestine.* A cannula of the type devised by Gaddum and modified by MacIntosh [1938] for perfusing the superior cervical ganglion in cats was tied into the peripheral end of the superior mesenteric artery which was divided near its origin. Perfusion was started at once with aerated Locke or Tyrode solution containing eserine in a concentration of 1 in 100,000 and at a temperature between  $37$  and  $39^{\circ}\text{C.}$  The upper and lower third of the small intestine were tied off and removed, leaving for perfusion a piece varying in length between 70 and 140 cm. A cannula was tied into the portal vein for collecting the venous outflow and into the lumen at each end of the perfused part the inside of which was washed out with about 50 c.c. of the perfusion fluid. After ligating all vascular connexions with the rest of the body the perfused part was transferred to a perfusion chamber which consisted of an 11 cm. glass funnel immersed in an electrically heated water-bath. An inverted Petri dish was fixed with plasticine into the inside of the funnel leaving room for fluid to run down the funnel. The perfused intestine was spread on the dish, avoiding kinking, and the perfusion cannula was fixed in position. The venous cannula was connected to a fine rubber tube and the venous effluent collected and assayed separately from the fluid oozing from the outside and inside of the intestinal wall which was collected by the funnel. The funnel was covered with a glass plate and a thermometer was placed in the chamber so obtained. Its temperature was kept well over  $30$  and below  $40^{\circ}\text{C.}$  The rate of inflow was 2–3 c.c./min. From the beginning of the perfusion to the collection of the first sample 30–40 min. elapsed.

The samples were assayed for acetylcholine on the frog's rectus muscle suspended in diluted eserinated Locke solution. The muscle was sometimes further sensitized by adding urethane to the solution [Emmelin, 1939]. The high choline content of the samples as well as the presence of another interfering principle made it necessary, in order to obtain the true equivalent for acetylcholine, to make the control solution not in saline but in part of the sample itself after its acetylcholine had been destroyed by NaOH and then again neutralized. After treatment with NaOH the sample was further assayed against choline on the frog rectus and the values obtained compared with the choline estimates found by acetylation.

## RESULTS

*The suspended fresh preparation of the intestine**Acetylcholine*

*In the presence of glucose.* The immediate shortening of the isolated gut caused by small doses of acetylcholine occurs even when both ends are closed and the inside pressure raised to 3 cm. of water. Usually the shortening is accompanied by a strong but evanescent contraction of the circular muscle starting at the upper end and frequently passing over the whole preparation. This causes a large reduction in volume lasting a few seconds, after which the volume returns to an intermediate position in which it remains as long as the shortening persists (see Figs. 3, 4). This remaining reduction in volume, if small, may be accounted for by the shortening, if pronounced, it may indicate some tonic effect on the circular muscle. In a few experiments the shortening appeared to proceed without any visible effect on the circular muscle, but even in these instances, after washing out the acetylcholine, the circular muscle usually passed through a period of increased activity. With larger doses of acetylcholine left in contact with the gut for several minutes, the contraction of the longitudinal muscle was well maintained but might be interrupted by slight evanescent lengthening of the gut as a result of a strong contraction of the circular muscle. This effect was never as pronounced as that produced by prolonged action of eserine or muscarine.

*In the absence of glucose.* The excitability of the longitudinal muscle to acetylcholine and other stimulating substances decreases and can be lost, whereas that of the circular is much better retained. The first sign of decreased excitability of the longitudinal muscle was its inability to remain contracted during the 60-90 sec. the acetylcholine was left in contact with the gut. During this period the muscle relaxed and sometimes returned to its original length. If rhythmic activity had been present prior to the acetylcholine it sometimes disappeared for a minute or two. In several instances this happened after the acetylcholine had been washed out. It was not associated with activity of the circular muscle. Later on the contractions of the longitudinal muscle to acetylcholine became smaller and eventually the muscle might fail to contract at all, stoppage of its spontaneous rhythm sometimes being the sole response. This stage was reached in some preparations more slowly than in others. Its appearance was accelerated by shortening the intervals between successive administrations of acetylcholine and by increasing their dose. On the other hand, there was usually some recovery by

lengthening the intervals. The preparation, a record of which is shown in Fig. 1, had been suspended for over an hour in a glucose-free Tyrode solution and subjected during the last 20 min. to repeated doses of  $1\text{ }\mu\text{g.}$  of acetylcholine at short intervals, the last two responses being shown at A and B. The contractions are not well sustained, and that at B is weaker and of shorter duration than that at A. After a rest of 5 min. acetylcholine caused strong and maintained contraction (at C), but recovery was evanescent as seen from the subsequent responses (D-F). An interval of 8 min. caused recovery (at G) which was more pronounced than that at C. The interpolation, once or twice, of a large dose of acetylcholine was even more effective in decreasing the excitability than was shortening the intervals. It usually resulted in a period of complete inexcitability

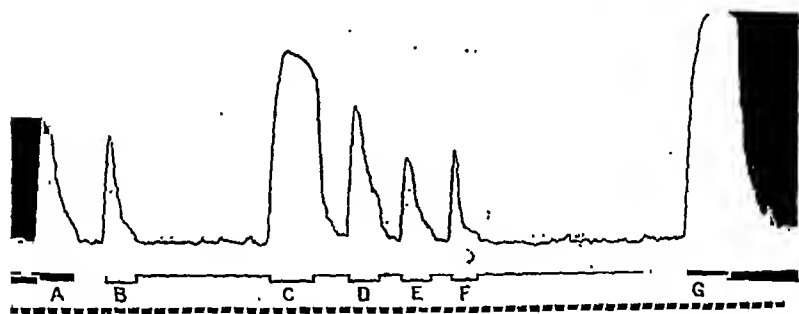


Fig. 1. Rabbit's intestine in 35 c.c. glucose-free Tyrode solution; lumen left open at both ends. A-G =  $1\text{ }\mu\text{g.}$  acetylcholine chloride. Time in 30 sec.

of the longitudinal muscle (see Fig. 5 A, B). The figure shows also that some recovery took place after a rest of over 10 min., when  $10\text{ }\mu\text{g.}$  of acetylcholine were given at C.

The absence of glucose from the Tyrode solution did not abolish the effect of acetylcholine on the circular muscle. It thus became possible to obtain records of contractions of the circular muscle without interference by shortening of the intestine. Relatively large doses were necessary to produce the effect, and it is therefore possible that the lack of glucose had diminished the excitability of the circular muscle. Small effects were obtained with  $5\text{--}10\text{ }\mu\text{g.}$  and large effects with  $50\text{--}100\text{ }\mu\text{g.}$  in a 45 c.c. bath (Figs. 2 A, 5 A-C). There was some reduction in the responses if large doses were given repeatedly at short intervals. Strong contractions of the circular muscle were often associated with lengthening of the gut

(Fig. 5 B), and if a contraction of the longitudinal was present it was cut short by the contraction of the circular muscle (Fig. 5 A).

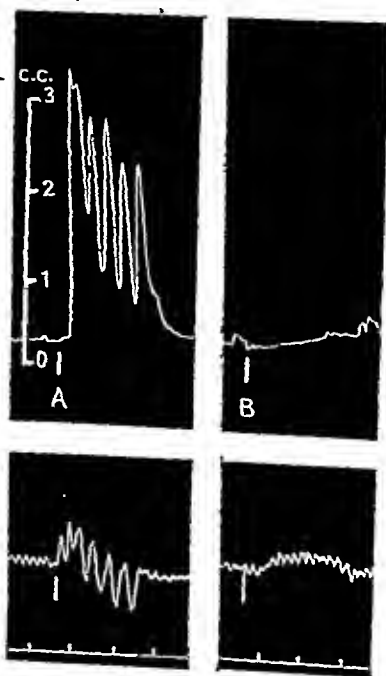


Fig. 2. Rabbit's intestine in 45 c.c. glucose-free Tyrode solution. A and B = 100  $\mu$ g. acetylcholine chloride for 1 min.; between A and B 5  $\mu$ g. of atropine. In this and the following figures the vertical scale indicates the changes in intestinal volume in c.c. Time in 30 sec.

### Muscarine

The fact that relatively large doses of acetylcholine were needed to contract the circular muscle in the absence of glucose might suggest a nicotine-like effect on the nerve cells. Its abolition by small doses of atropine (see later) would be no evidence against this interpretation. We have therefore, at the suggestion of J. H. Gaddum, examined the effect of muscarine which is devoid of a nicotine-like action. It stimulated the longitudinal as well as the circular muscle. In the absence of glucose the effect on the longitudinal but not on the circular muscle became weaker and ultimately disappeared, the factors favouring this condition being the same as those described for acetylcholine. On the circular muscle muscarine had a relatively greater effect than acetylcholine which, in the presence of glucose, became apparent when large doses were compared.

In Fig. 3 the effects on the two muscle layers of 1  $\mu$ g. of acetylcholine (A) and of 0.2 c.c. of 1 in 400 of the solution of muscarine at our disposal (B) correspond approximately. D and E are the effects of 10 and 50  $\mu$ g. of acetylcholine respectively, and C that of a dose of muscarine 20 times stronger than that given at B. It causes a greater reduction in volume than 50  $\mu$ g. acetylcholine. The contraction of the ring muscle is so strong as to produce lengthening of the gut which subsides after washing out the muscarine. In observing the preparation the impression is gained

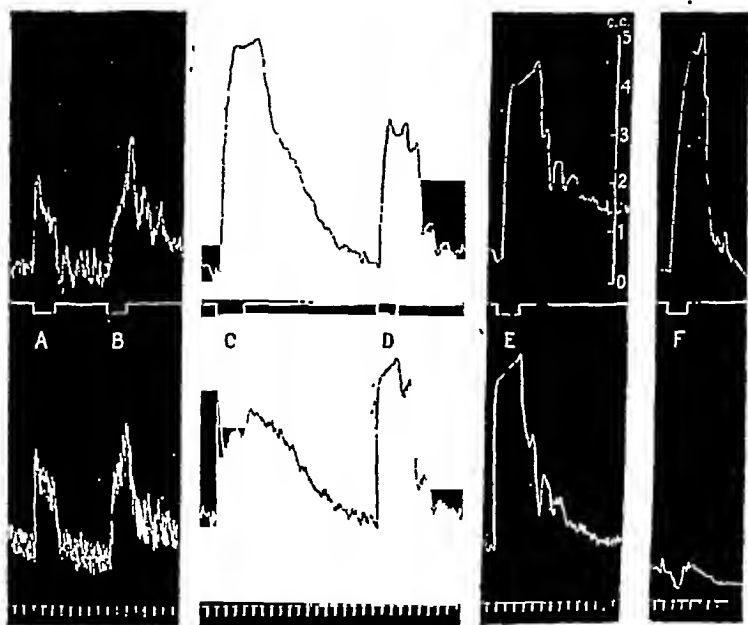


Fig. 3. Rabbit's intestine in 60 c.c. Tyrode solution. A, D and E = 1, 10 and 50  $\mu$ g. acetylcholine chloride. B, C and F = muscarine. F is from a different preparation in glucose-free Tyrode solution. Time in 30 sec.

that the lengthening is brought about passively by the contraction of the circular muscle. Trendelenburg [1917] observed similar changes in the two muscle layers, when the pressure in the lumen of the intestine was raised, but he attributed the lengthening to active inhibition of the longitudinal muscle co-ordinated with the strong contraction of the circular muscle.

Direct observation of the preparation also showed that muscarine was more active on the circular muscle than was acetylcholine. At C the circular muscle became completely contracted occluding the lumen of the

whole piece of gut. The effect at F was obtained with twice the dose of muscarine given at C, but on a different preparation suspended in Tyrode solution free from glucose. There was no stimulation of the longitudinal muscle which lengthened during contraction of the circular muscle. It was not possible, in the absence of glucose, to obtain contractions of the circular muscle as strong as those produced by muscarine even by increasing the dose of acetylcholine to several 100  $\mu$ g. On the other hand, if the excitability of the longitudinal muscle was partly depressed, acetylcholine often caused a relatively strong contraction of this muscle, whereas that produced by muscarine was small or absent. The effect of muscarine on the longitudinal muscle appears, therefore, to be relatively weaker than that of acetylcholine.

### *Eserine*

*In the presence of glucose.* Fig. 4 shows the interaction of the stimulating effect of eserine on the two muscle layers. Stimulation starts after a latency which varied between 10 and 30 sec. and the contraction proceeds gradually. With small doses of eserine even longer latencies were observed. The stimulation of the circular muscle usually began a few seconds later and overcame the shortening of the gut. The figure illustrates the sudden onset of periods of maximal contractions of the circular muscle spreading over the whole wall and causing lengthening of the gut which shortens again with partial relaxation of the circular muscle. The interaction of the two muscles repeated itself four times, the last occurring after the eserine had been washed out. Sometimes the circular muscle remained strongly contracted as long as the eserine was left in contact. This caused a trough-like depression on the record of the longitudinal muscle.

*In the absence of glucose.* The stimulating effect of eserine on the longitudinal muscle was weak or absent, whereas that on the circular muscle was retained (Fig. 5). The latency appeared to be longer and the contraction disappeared more quickly after washing out the eserine, indicating some impairment in the functioning of the circular muscle.

### *Glucose*

*Effect on the longitudinal muscle in the absence of glucose.* A preparation suspended with the lumen left open at both ends relaxed progressively for 20-60 min. There was usually first an increase in the amplitude of the rhythmic contractions due to more complete relaxation followed by a decrease in amplitude due to less extensive contraction. In addition, the



tone diminished independently of these changes. Glucose added to the relaxed preparation had a stimulating effect and restored the excitability of the muscle to subsequent administration of acetylcholine. The stimulation by glucose started after a latency of 15–60 sec., the rhythmic movements increased in amplitude but not in frequency and the tone

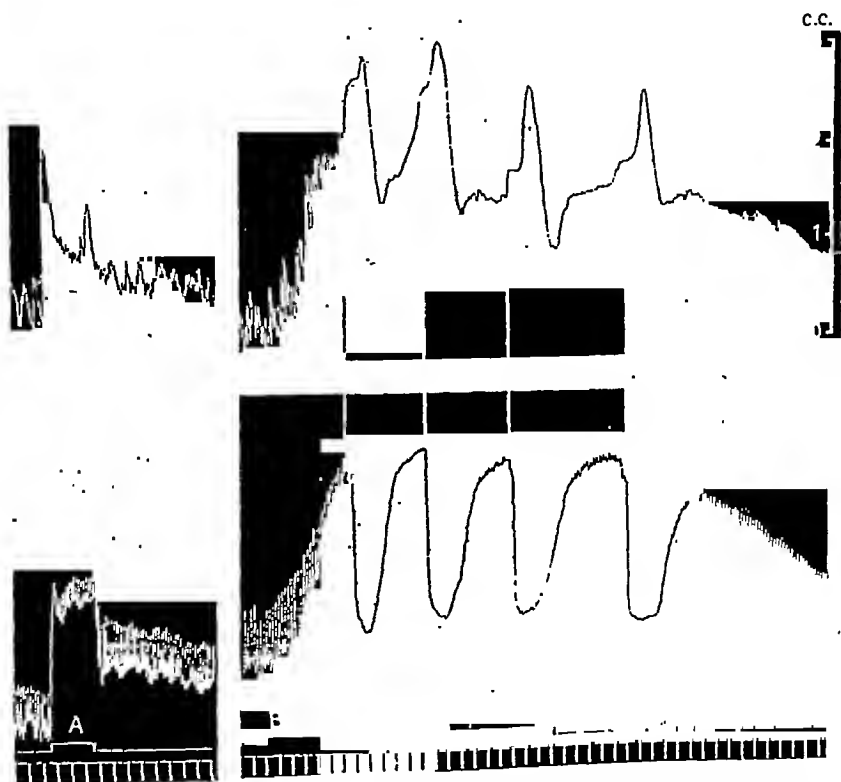


Fig. 4. Rabbit's intestine in 45 c.c. Tyrode solution. A = 1  $\mu$ g. acetylcholine chloride; B = 10  $\mu$ g. eserine sulphate. The four vertical white lines indicate corresponding points on the upper and lower tracings. Time in 30 sec.

became stronger. During the latency there was often some inhibition in tone and activity. Small stimulating effects consisting of an increase in activity only were sometimes observed on the addition of 1–2 mg. of glucose to a 35 c.c. bath. In some preparations, particularly from the lower end of the ileum, stimulation affected mainly the rhythmic activity, in others the effect was mainly on the tone (Fig. 6). The stimulating effect increased with repeated administration of glucose, the first doses often

increasing activity only, the later ones the tone (Fig. 7). It will be seen from both figures that when only the rhythmic activity was stimulated, the shortening increased with each rhythm, but the base-line was not affected. In the experiment of Fig. 7 the first dose of glucose was given

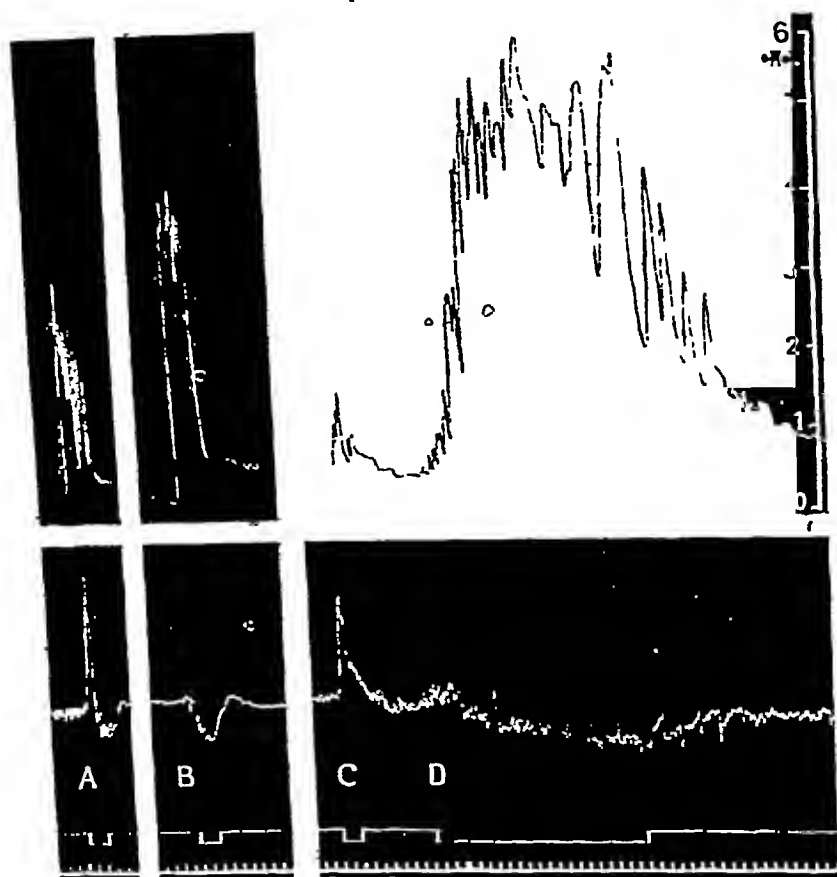


Fig. 5. Rabbit's intestine in 60 c c. glucose-free Tyrode solution. A, B and C = 50, 100 and 10  $\mu$ g. acetylcholine chloride; D = 20  $\mu$ g. eserine sulphate. Time in 30 sec.

25 min. after the preparation had been suspended. In preparations suspended for a longer time even a first dose of glucose produced a strong stimulating effect, but some increase in the response was obtained with repeated administration.

The restoration of the excitability of the muscle by glucose was usually studied with acetylcholine, but in some instances choline and pilocarpine were used with identical results. The excitability remained

elevated for some time after the glucose had been washed out, and the contractions with acetylcholine were not only more powerful but, also better maintained (Fig. 8). This change in excitability could be obtained with amounts of glucose which had scarcely any direct stimulating effect. The period of recovery varied in length in different preparations; increase

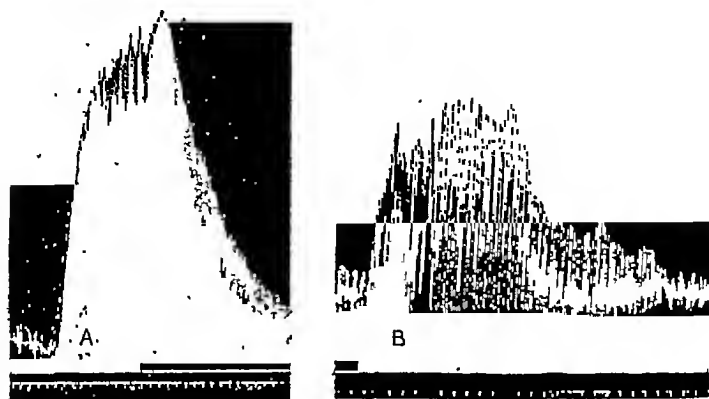


Fig. 6. Rabbit's intestine in 35 c.c. glucose-free Tyrode solution, lumen left open at both ends. Two preparations from different animals. A and B = 20 mg. glucose; latency at A 18, at B 15 sec. Time in 30 sec.

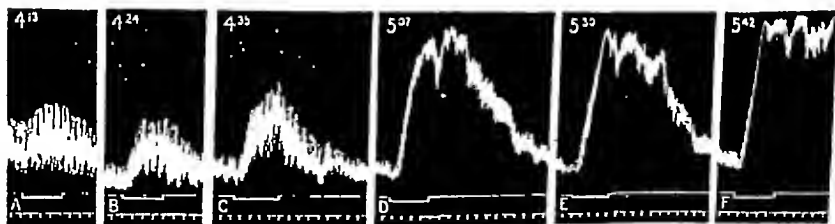


Fig. 7. Rabbit's intestine suspended at 3.50 p.m. in 35 c.c. glucose-free Tyrode solution; lumen left open at both ends. A-F = 10 mg. glucose; the latencies at C-F were 21, 21, 22 and 14 sec. On top of tracing, time when glucose was given. Time in 30 sec.

in the dose of glucose and in the time it was left in contact with the gut lengthened it, shortening the intervals between successive doses of acetylcholine and increasing their dosage shortened it.

Closing the lower end of the preparation did not affect the results, but when both ends were closed and the inside pressure raised to  $2\frac{1}{4}$ –3 cm. of water the stimulating effect of glucose diminished (Fig. 9 A, B) and, with repeated administration, it decreased further and sometimes dis-

appeared. In the experiment of Fig. 10 the longitudinal muscle had become insensitive to the stimulating effect of glucose (at C) following its repeated administration, but it was still effective in restoring the excitability of the muscle to acetylcholine. When in this condition glucose was given in the presence of a weak concentration of acetylcholine in the bath or vice versa, a strong and sustained contraction of the longitudinal muscle occurred. In Fig. 10, for instance, 5  $\mu$ g. of acetylcholine (B) or 50  $\mu$ g. glucose (C) when given separately, were ineffective, but when the acetylcholine was given with or after the glucose a strong contraction was obtained. Previous to the glucose even 100  $\mu$ g. of acetylcholine (A) had no effect on the longitudinal muscle. The restoration of excitability without direct stimulation of the muscle by glucose therefore may have been due to the fact that the acetylcholine continuously liberated in the



Fig. 8. Rabbit's intestine suspended at 11.00 a.m. in 14 c.c. glucose-free Tyrode solution. Lumen left open at both ends. B-E, G-M = 1  $\mu$ g. acetylcholine chloride. F = 5 mg. glucose, latency 35 sec. Time in 30 sec.

intestinal wall did not reach a concentration sufficient for stimulation under these conditions. According to this assumption raising the concentration should bring back the stimulating action of glucose. This can be achieved by eserine. If given with a non-stimulating dose of glucose it causes after a latency of 20-50 sec. a slowly progressing but strong contraction of the longitudinal muscle, and its relaxation proceeds very gradually after washing out the eserine and glucose.

*Effect on the circular muscle in the absence of glucose.* The stimulating effect was small and irregular. The shortening of the gut was associated with a reduction in volume which was often so small (Fig. 9) as to be wholly accounted for by it. If the reduction was stronger it may have resulted from an increase in tone of the circular muscle. There was usually some increased activity after the glucose had been washed out and the longitudinal muscle had relaxed. A few rapid but strong contractions of the whole circular muscle sometimes occurred leading to lengthening of



Fig. 9. Rabbit's intestine in 35 c.c. glucose-free Tyrode solution; lumen closed at lower end and at upper end after A. A and B = 75 mg. glucose, latencies 10 and 20 sec. C = 400 mg. sodium lactate. Time in 30 sec.

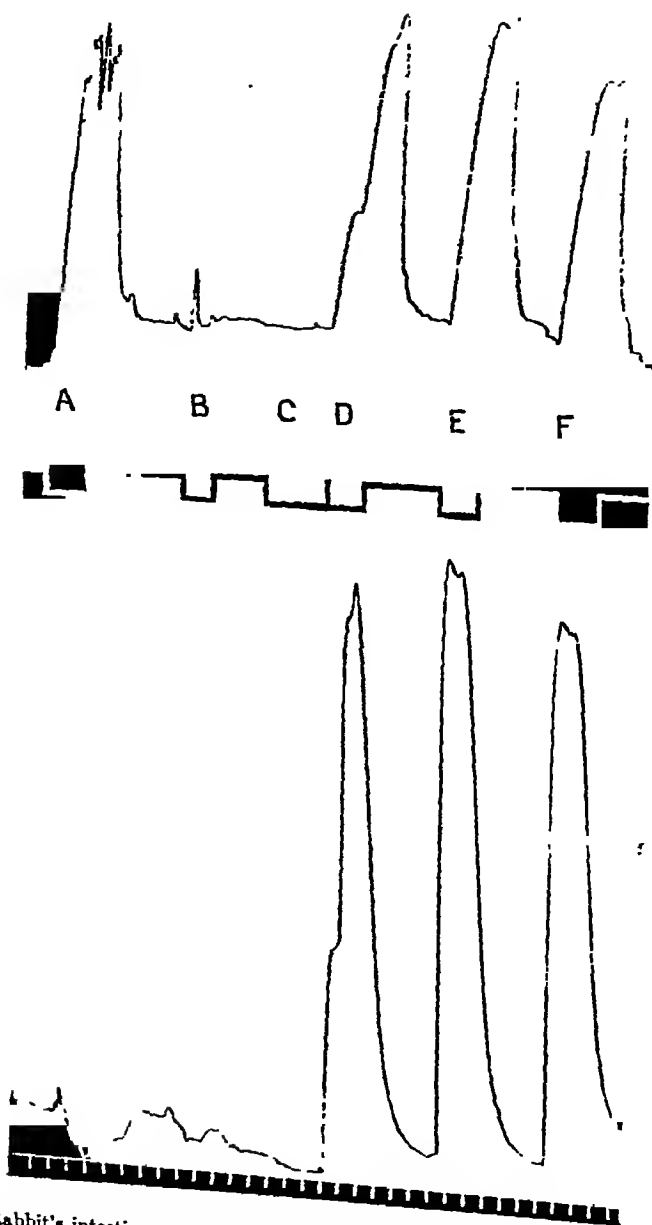


Fig. 10. Rabbit's intestine in 35 c.c. glucose-free Tyrode solution. A=100  $\mu$ g.; B, D, E and F = 5  $\mu$ g. acetylcholine chloride. C = 50 mg. glucose for 3 min., D given after the second minute. Time in 30 sec.

the gut. There were occasionally isolated constrictions on different parts of the circular muscle, and in a few experiments regular rhythmic contractions of the whole layer at a frequency of 9–11 per min. occurred for 2 to 3 min. either before the shortening or after the relaxation of the longitudinal muscle had taken place (Fig. 9 B).

*Effect in the presence of glucose.* On a preparation suspended in Tyrode solution containing its normal 0.1% glucose the addition of enough glucose to double or treble its concentration in the bath caused no stimulation. It usually produced slight immediate inhibition in tone and activity. Further increase in the concentration of glucose caused greater inhibition which might be followed by slight stimulation of the muscle (Fig. 11 C). Direct observation of the preparation through the glass walls of the bath revealed the absence of contractions of the circular muscle. The effects on the longitudinal muscle were not due to an increase in tonicity of the bath fluid, since they occurred also when glucose was added in an appropriate volume of NaCl-free Tyrode solution in which the NaCl had been replaced by equimolecular amounts of glucose.

#### *Other monosaccharides*

The following sugars have been tested on the longitudinal muscle, no volume record being taken:

*Mannose* has a stimulating action, in the absence of glucose from the bath, and restores the excitability of the muscle to acetylcholine. The response increases with repeated administration. Mannose is about 25–30% as active as glucose. Stimulation starts after a longer latent period and reaches its maximum more slowly than after glucose. If kept in contact with the gut for 1–2 min. the effect reaches its maximum after the mannose has been washed out. In one experiment the latent period following 2.5 mg. of glucose was 27 sec., that following a similarly effective dose of 10 mg. of mannose was 37 sec. In another similar experiment the latent periods were 16 and 26 sec. respectively. As with glucose there was usually some immediate inhibition.

On preparations suspended in Tyrode solution containing glucose, mannose caused immediate inhibition, sometimes followed by some stimulation (Fig. 11 A) which was a little stronger than that caused by glucose in this condition.

*Galactose* had a weak stimulating action on preparations suspended in glucose-free Tyrode solution. The effect of 100 mg. corresponded to that of 2–3 mg. of glucose. Stimulation was often preceded by slight immediate inhibition, and after washing out the galactose the muscle

showed a period of increased excitability to acetylcholine. On preparations suspended in Tyrode solution containing glucose, galactose had an immediate inhibitory effect only.

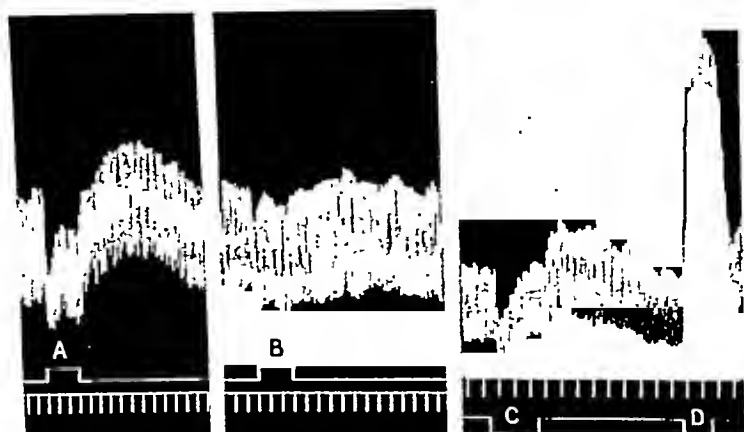


Fig. 11. Rabbit's intestine in 14 c.c. Tyrode solution; lumen left open at both ends. A=100 mg. mannose; B=100 mg. lactose; C=100 mg. glucose; D=1  $\mu$ g. acetylcholine chloride. Time in 30 sec.

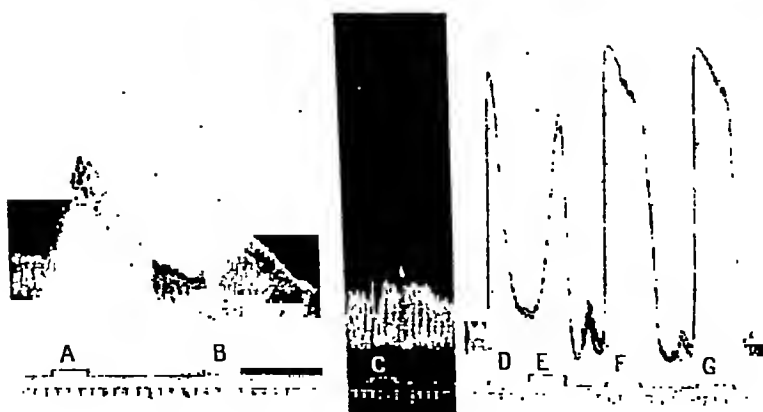


Fig. 12. Rabbit's intestine in 14 c.c. glucose-free Tyrode solution; lumen left open at both ends. A = 5 mg. glucose; B, C and E=100 mg. laevulose; D, F and G=10  $\mu$ g. acetylcholine chloride. From C to G a new piece of intestine from same animal. E added without washing out the acetylcholine. Time in 30 sec.

*Laevulose* had a slight stimulative action on preparations suspended in glucose-free Tyrode solution followed by a period of increased excitability to acetylcholine. In the experiment of Fig. 12 the effect of 5 mg.



of glucose (A) was compared with that of 100 mg. of laevulose (B). During the latent period there was some inhibition. The muscle which had been unable to maintain the contraction caused by acetylcholine regained this property through the addition of laevulose to the bath. The contraction at D was maintained for 15 sec. only, although the acetylcholine was not washed out. 100 mg. of laevulose, which alone had a very slight stimulating effect (C), caused, in the presence of the acetylcholine, a strong contraction after a latency of about 20 sec. (E). The excitability remained elevated during the next minutes (F and G).

On preparations suspended in Tyrode solution containing glucose laevulose caused immediate inhibition which was not followed by stimulation.

### *Disaccharides*

*Maltose* and *lactose* had no stimulating action if added in doses up to 100 mg. to a 14 c.c. bath containing no glucose. The addition of 200 mg. of maltose produced slight inhibition followed by slight stimulation which was less than that produced by 2 mg. of glucose and might have been due to glucose in the preparation as impurity. Lactose had no effect in restoring the excitability of the muscle to acetylcholine. No corresponding experiments were carried out with maltose. If tested in the presence of glucose, lactose caused slight immediate inhibition (Fig. 11 B).

### *Sodium pyruvate*

On preparations suspended in glucose-free Tyrode solution sodium pyruvate has a strong stimulating effect on the longitudinal muscle and restores its excitability to acetylcholine. On the circular muscle stimulation is weak and irregular. The effect on the longitudinal muscle differs from that of glucose in that the latency is shorter and relaxation after washing out the bath more rapid. In all other details the effect resembles that of glucose. In the experiment of Fig. 13 the latencies of two doses of 1 mg. of pyruvate (A and F) were 8 and 9 sec., whereas those of equipotential doses of glucose (B and E) were 20 and 16 sec. respectively. Compared weight for weight sodium pyruvate was 10-30 times as active as glucose.

In the presence of glucose, pyruvate had on the longitudinal muscle a slight stimulating effect which was a little stronger than that of glucose.

### *Sodium lactate*

*Effect in the presence of glucose.* Lactate had a stimulating action on both muscle layers. In a preparation suspended in such a way as to leave both ends open the contraction of the circular muscle sometimes caused

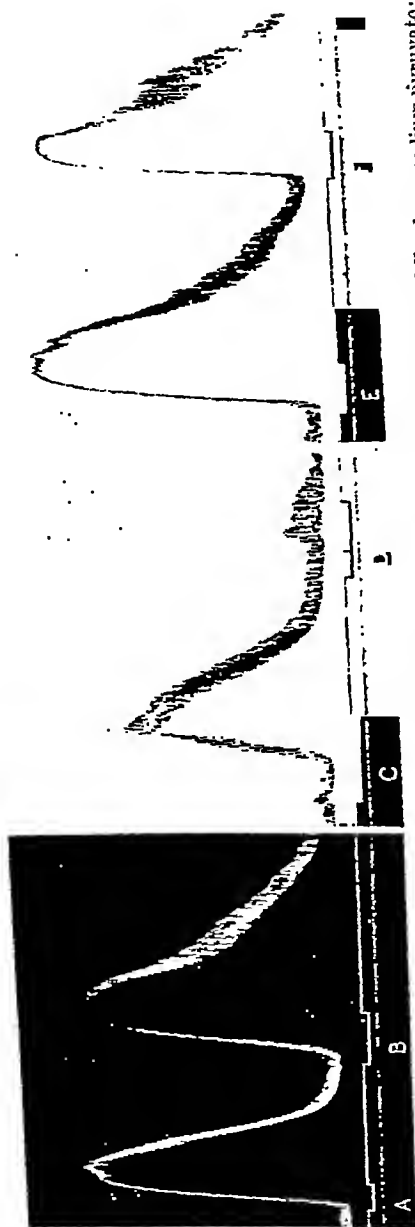


Fig. 13. Rabbit's intestine in 14 c.c. glucose-free Tyrode solution; lumen left open at both ends. A and F = 1 mg. sodium pyruvate; B and F = 15 mg. glucose; C and D = 5 mg. phloridzin for 3 min. followed after the first minute by 1 mg. sodium pyruvate (C) and 15 mg. glucose (D). Time in 30 sec.

expulsion of great amounts of excreted juice through the lower end producing frothing and interference with the action of lactate. It was therefore necessary even when the effect on the longitudinal muscle alone was recorded to tie the lower caecal end over the glass cannula connected with the rubber tube to the aspirator (see 'Method'). The contents of the gut then emptied into the cannula.

The doses of sodium lactate necessary to produce strong stimulating actions were so great as to increase the tonicity of the solution. For instance, the addition to a 45 c.c. bath of 150 mg. of sodium lactate, which is equimolecular to 79 mg. of NaCl, represented an increase in salt concentration of about 20%. Miss M. Vogt [1942], who had found that such changes in salt concentration caused strong stimulating effects on both muscle layers, drew our attention to the fact that responses to lactate injections without correction of the salt factor might be accounted for partly by an increase in salt concentration. In the following experiments lactate, therefore, was added to the bath in the appropriate volume of NaCl-free Tyrode solution, the NaCl being replaced by equimolecular amounts of sodium lactate.

The stimulating effect on the longitudinal muscle of increasing doses of lactate is seen in Fig. 14. Stimulation started after a latency of a few seconds. The addition to the 45 c.c. bath of 40 mg. of sodium lactate or less had no longer a stimulating effect. After closing the upper end of the preparation and raising the inside pressure of the gut to  $2\frac{1}{2}$  cm. of water the shortening effect of large doses of lactate was somewhat diminished. In some experiments the shortening was interrupted by strong contractions of the circular muscle layer causing lengthening of the gut beyond its original level. In the experiment of Fig. 14, at D, the lengthening occurred before washing out the lactate and was associated with a strong contraction of the circular muscle. At B the gut volume is recorded simultaneously; the sudden lengthening is associated with a great reduction in volume due to contraction of the whole circular muscle layer.

*Effect in the absence of glucose.* Whereas the absence of glucose did not appear to modify the stimulating effect of lactate on the circular muscle that on the longitudinal one was reduced or even abolished. In the experiment of Fig. 15 the small effect obtained with 300 mg. of lactate (at B) is contrasted with the strong stimulation of the longitudinal muscle by glucose. The lactate did not appear to modify the response of the muscle to subsequent administration of acetylcholine, but the response to glucose became reduced for some time. This is seen by a comparison of the responses to glucose at A, C and D in Fig. 15. After successive

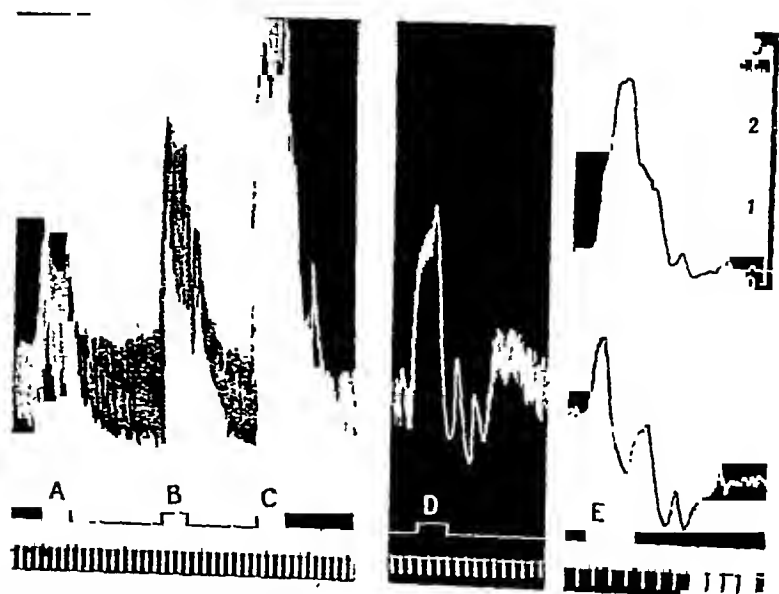


Fig. 14. Two preparations of rabbit's intestine in 45 c.c. Tyrode solution. Lower lumen closed; upper lumen closed after D. A = 75 mg., B, D = 150 mg. and C, E = 300 mg. sodium lactate. D and E the second preparation. Time in 30 sec.

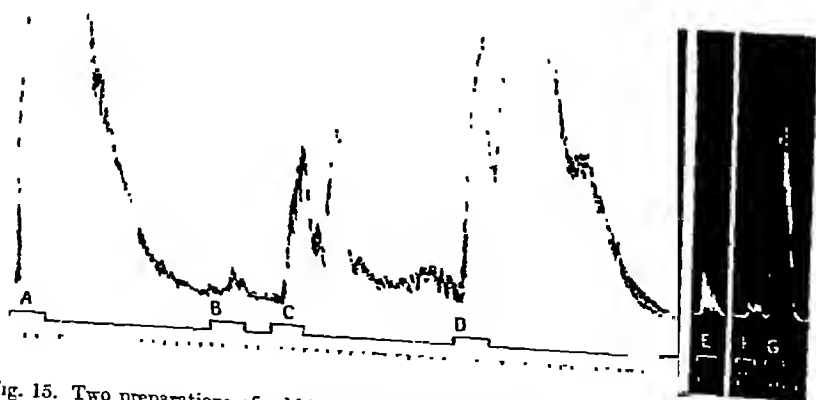


Fig. 15. Two preparations of rabbit's intestine in 45 c.c. Tyrode solution. Lower lumen closed. A, C, D and F = 50 mg. glucose; latency at A 18, at C 42 and at D 34 sec. B = 300 mg., E and G = 150 mg. sodium lactate. E, F and G the second preparation. Time in 30 sec.

administration of large doses of lactate the longitudinal muscle sometimes showed a period of complete insensitivity to the stimulating action of glucose, its sole effect then being an increase in excitability to stimulating substances such as acetylcholine. In this condition lactate also regained its stimulating action on the longitudinal muscle. This is shown by the difference in the response to lactate at E and G in experiment of Fig. 15. The stimulating action of glucose which, previous to the lactate, had been pronounced was nearly abolished when given at F after three successive doses of lactate. Sometimes lactate in the course of the experiment acquired a stimulating action on the longitudinal muscle without any addition of glucose to the bath. Having for six or seven injections stimulated the circular muscle only, the lactate from the next injection onwards suddenly stimulated strongly both muscle layers.

On fresh preparations the stimulating effect of lactate on the circular muscle usually consisted of more or less regular rhythmic contractions of the whole wall at a frequency of about seven per minute. These could be observed through the glass wall of the bath and were responsible for the rhythmic changes seen in the volume record of Fig. 16. In preparations, which had been suspended for a long time and had become tired the effect of lactate on the circular muscle resulted often in a more sustained tonic contraction which lasted until the lactate had been washed out.

### *Phloridzin*

*Glucose.* Phloridzin inhibits the stimulating action of glucose on the longitudinal muscle (Fig. 13 D), but it appears to have no direct action on the gut itself. Therefore on a relaxed preparation suspended in glucose-free Tyrode solution it has no effect or only slightly inhibits the rhythmic contractions. On such a preparation it has also no influence on the response to acetylcholine. When the phloridzin has been washed out complete recovery of the stimulating action of glucose ensues (Fig. 13 E). The inhibition of the stimulating action by phloridzin is responsible for the depression produced on preparations suspended in glucose containing Tyrode solution. In Fig. 17 A and B the phloridzin depresses mainly the tone, and at C the rhythmic movements. The recovery between the two administrations of 30 mg. of phloridzin at A and B resembles the stimulating action which glucose produces in the experiment of Fig. 6 A, and the inhibition of the rhythmic movements at C represents a reversal of the stimulating effect of glucose such as shown in Fig. 6 B.

*Sodium pyruvate.* Its stimulating action is also inhibited by phloridzin but less than that of glucose. Fig. 13 shows the effect of 5 mg. of phloridzin

on equipotential doses of glucose and pyruvate. The response to glucose is almost abolished (D), whereas pyruvate is still highly active (C). However, the contraction is smaller, starts after a longer latency and

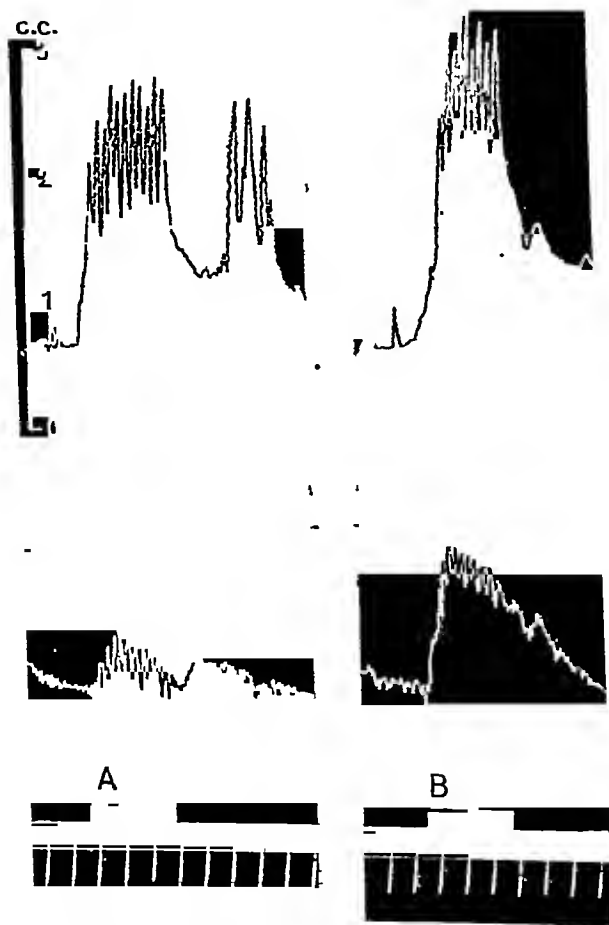


Fig. 16. Rabbit's intestine in 45 c.c. glucose free Tyrode solution.  
A = 150 mg, B = 300 mg. sodium lactate. Time in 30 sec.

proceeds more slowly than that produced by pyruvate without phloridzin (A and F). Larger doses of phloridzin completely inhibit the action of pyruvate.

*Sodium lactate.* Phloridzin does not affect the strong stimulating action on the circular muscle and does not inhibit the stimulation of the

longitudinal muscle when observed in the absence of glucose from the bath.

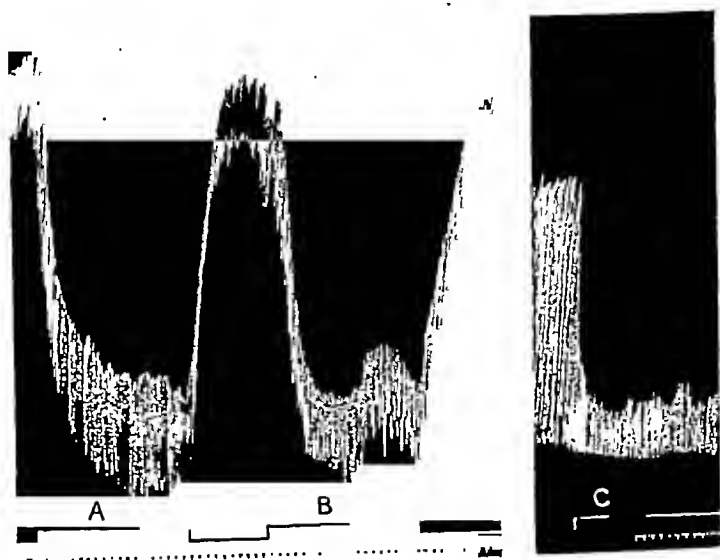


Fig. 17. Rabbit's intestine in 35 c.c. Tyrode solution; lumen left open at both ends. A and B = 30 mg., C = 20 mg. phloridzin. C, preparation from different rabbit. Time in 30 sec.

### Atropine

*Effect in the absence of glucose.* In the beginning of an experiment, while the longitudinal muscle is slowly relaxing, atropine (10–50  $\mu$ g. to a 45 c.c. bath) may cause rapid relaxation. At later stages the inhibitory effect of atropine is slight and evanescent and may even be absent. Rhythmic contractions of the longitudinal muscle if present continue although added acetylcholine has no stimulating effect.

*Glucose.* During the stimulating action of glucose atropine causes rapid and sometimes complete relaxation of the longitudinal muscle, the spontaneous activity of which may cease (Fig. 18). After a few minutes rhythmic activity and tone reappear even when the atropine remains in the bath and although the muscle remains insensitive to added acetylcholine. Atropine thus produces a trough-like depression on the tracing (Fig. 18 B). When atropine is given before glucose the stimulation is delayed and diminished but not abolished, even though added acetylcholine is ineffective.

*Acetylcholine and muscarine.* Small doses of atropine abolish the

stimulating action of acetylcholine and muscarine not only on the longitudinal but also on the circular muscle (Fig. 2).

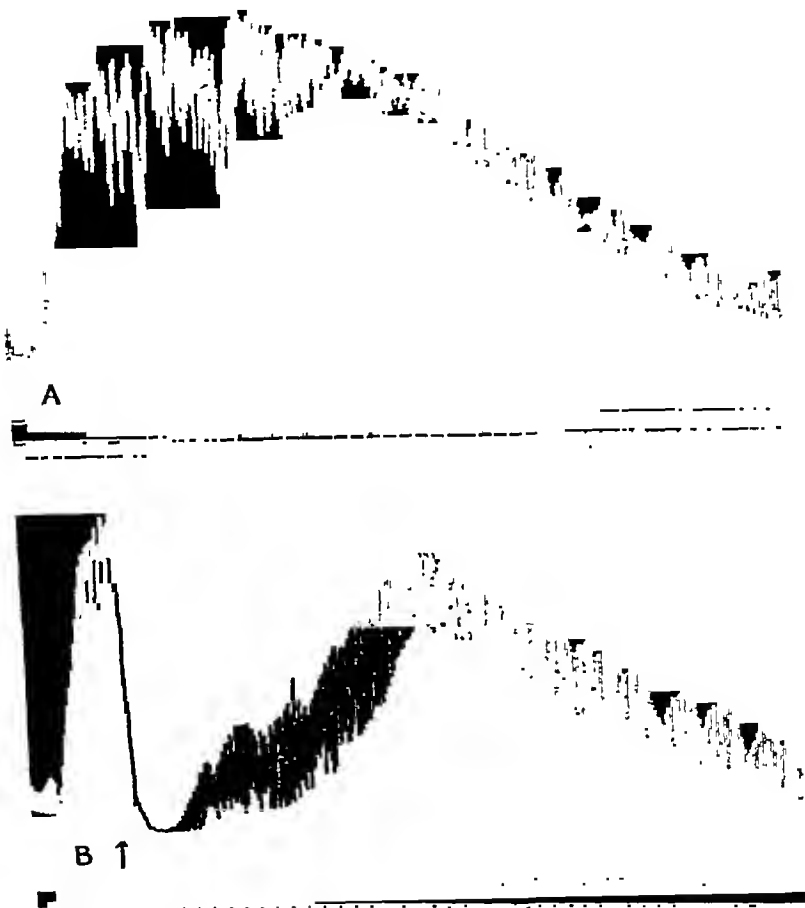


Fig. 18. Rabbit's intestine in 35 c.c. glucose-free Tyrode solution; both lumen left open. A and B = 20 mg. glucose for 6 min. At the arrow 50  $\mu$ g. atropine until end of tracing. Time in 30 sec.

#### *The suspended cooled preparation of the intestine*

A preparation suspended in glucose-free Tyrode solution after having been kept in such a solution at  $-1^{\circ}$  C. for 24-72 hr. reacts differently from a freshly suspended preparation. Apart from the fact that the



longitudinal muscle when observed in the absence of glucose from the bath.

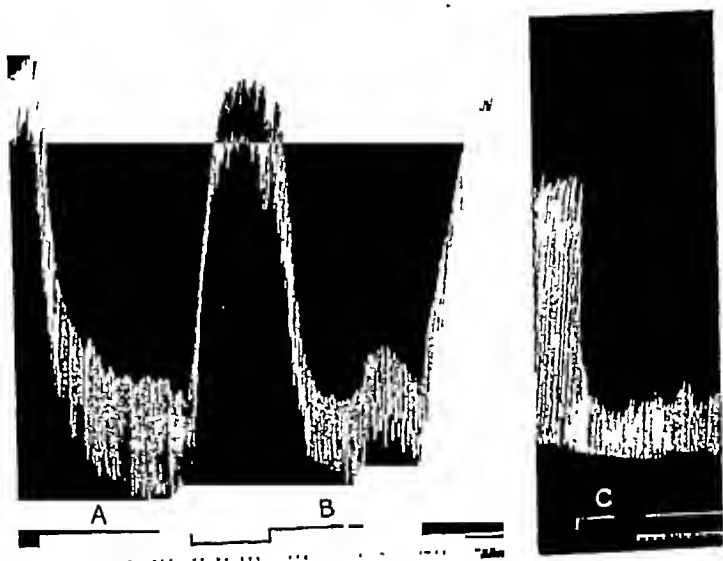


Fig. 17. Rabbit's intestine in 35 c.c. Tyrode solution; lumen left open at both ends. A and B = 30 mg., C = 20 mg. phloridzin. C, preparation from different rabbit. Time in 30 sec.

### *Atropine*

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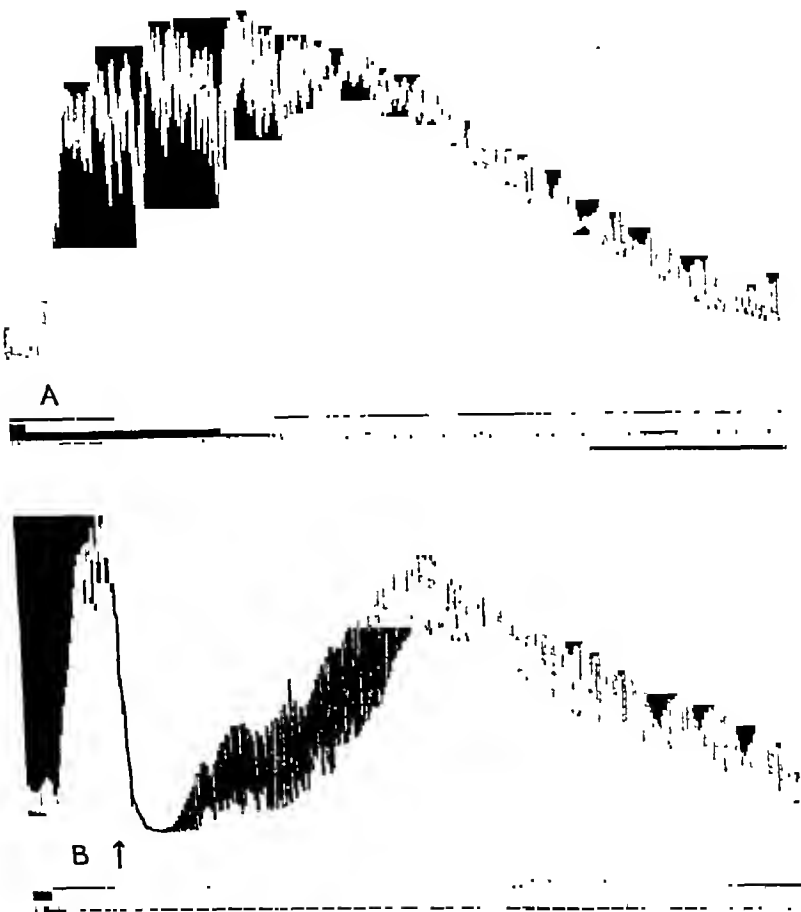


Fig. 18. Rabbit's intestine in 35 c.c. glucose-free Tyrode solution; both lumen left open. A and B = 20 mg. glucose for 6 min. At the arrow 50  $\mu$ g. atropine until end of tracing. Time in 30 sec.

*The suspended cooled preparation of the intestine*

A preparation suspended in glucose-free Tyrode solution after having been kept in such a solution at  $-1^{\circ}$  C. for 24-72 hr. reacts differently from a freshly suspended preparation. Apart from the fact that the

spontaneous activity is weak or even absent it responds differently to acetylcholine, eserine and glucose. Roughly speaking the responses resemble those obtained on fresh preparations suspended in Tyrode solution containing glucose.

The sensitivity to acetylcholine is of the same order as that of fresh preparations, but it is difficult to render the muscle inexcitable by repeated administration of acetylcholine. The muscle remains contracted as long as the acetylcholine is in contact with the gut (Fig. 19 A, D); even after large doses contraction is at least partially retained (Fig. 19 E, H), and when they are given at short intervals no intensification of the state of

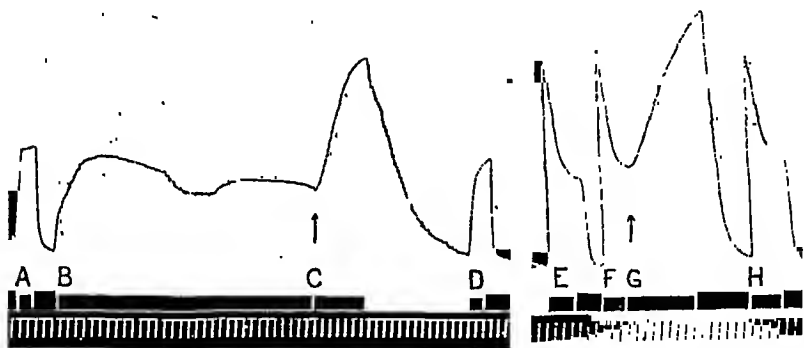


Fig. 19. Rabbit's intestine in 14 o.o. glucose-free Tyrode solution: cooled for over 72 hr.; both lumen left open. A and D = 2  $\mu$ g.; E, F and H = 5  $\mu$ g. acetylcholine chloride. B = 10  $\mu$ g. eserine sulphate. C = 30 mg. glucose without washing out eserine. G = 30 mg. glucose without washing out acetylcholine. Time in 30 sec.

decreased excitability is brought about. Only when successive doses are given without washing out the previous ones relaxation of the muscle may occur. The response to eserine also shows the difficulty of rendering the longitudinal muscle inexcitable despite the absence of glucose from the bath. The longitudinal muscle of the fresh preparation, in this condition, does not contract at all to eserine or contraction is maintained for a few seconds only. On the cooled preparation eserine causes a sustained contraction (Fig. 19 B), although the effect is weaker and more delayed than that of a similar dose on a fresh preparation in the presence of glucose.

On the cooled preparation glucose had no stimulating action, large doses causing some immediate inhibition of tone. Cooling a preparation for 4-5 hr. did not abolish the stimulating effect but reduced it. After

cooling for 12 hr. or longer stimulation was no longer obtained. But even on such a preparation it became possible to demonstrate the effect if the glucose was given during an eserine or acetylcholine contraction. In the experiment of Fig. 19 the cooled gut had been suspended for over 2 hr. in glucose-free Tyrode solution. During this period glucose, given in doses of 25–30 mg., never caused contraction although the muscle responded well to 0.2  $\mu$ g. of acetylcholine. At B 10  $\mu$ g. of eserine were given. After 16 min., during which the muscle remained contracted, 25 mg. of glucose were given (C) without washing out the eserine; a further contraction ensued starting after a latency of about 20 sec. and continuing until the glucose and eserine were washed out. At G is seen the effect of glucose during an acetylcholine contraction. In this experiment glucose did not increase the excitability of the muscle to subsequent doses of acetylcholine. In others excitability increased, but the effect was small and of short duration.

The cooled preparation in its reactions to eserine and acetylcholine did not behave like a muscle deficient of glucose, and in that case no stimulating action of glucose might be expected. Even a fresh preparation has to be suspended for some time in glucose-free solution before glucose exerts its stimulating effect. The powerful rhythmic contractions during this period apparently lead to the gradual depletion of the energy stores of the longitudinal muscle. It seemed possible that this condition was never really attained in the cooled preparation where there is only weak rhythmic activity. To test this possibility we tried to deplete a fresh preparation of its energy stores before cooling it. In the experiment of Fig. 20 a piece of intestine was suspended in glucose-free Tyrode solution (first column of the figure). After about 1 hr., 50 mg. of glucose were added for 1 min. (A). The strong contraction which raised the lever beyond the upper border of the tracing started after a latency of 25 sec. When the muscle had again relaxed the preparation was placed in a glucose-free Tyrode solution at  $-1^{\circ}$  C. and kept there for over 24 hr. when it was re-examined. Acetylcholine did not produce sustained contractions of the longitudinal muscle and the responses decreased with repeated administration. Glucose had retained its stimulating action although the response was altered. There was a latency of several minutes during which slight inhibition occurred, the contraction proceeded more gradually and lasted much longer. The contraction following the first dose of glucose again had raised the lever beyond the upper border of the tracing, but the response diminished with subsequent administration of glucose, the effect of the third dose being shown at B. The prolongation

of the response is no new phenomenon. Even on a fresh preparation the stimulating action of glucose in time becomes more prolonged, particularly after repeated administration. The long latency may be explained by the fact that the amounts of acetylcholine and choline liberated from a cooled preparation are relatively small, since the latency was at once shortened when the glucose was given in the presence of a weak concentration of acetylcholine or eserine. In the experiment of Fig. 20 the contractions to 1  $\mu$ g. of acetylcholine were not maintained and that at D was weaker than that at C. Without washing out the acetylcholine 50 mg. of glucose were added at E; the ensuing quick contraction started after a latency of 15 sec. The glucose and acetylcholine were washed out after 1 min.,

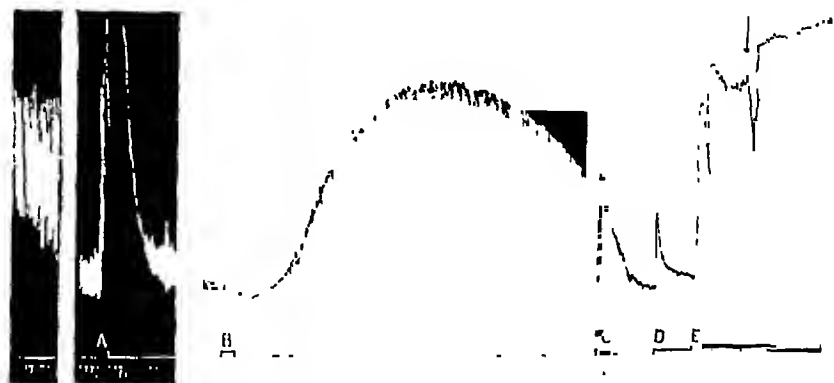


Fig. 20. Rabbit's intestine in 60 c.c. glucose-free Tyrode solution; cooled after A for over 24 hr. A, B and E = 50 mg. glucose for 90 sec. C and D = 1  $\mu$ g. acetylcholine chloride; the acetylcholine at D till end of tracing. At the arrow, washing. Time in 30 sec.

causing evanescent relaxation until fresh acetylcholine was added to the bath. It was more than 40 min. before the muscle had relaxed again. During the whole time the acetylcholine concentration in the bath was kept constant. Eserine had a weak stimulating action on this preparation, and glucose given during its presence caused a strong contraction of the longitudinal muscle after a latency of 15 sec.

#### *Perfusion of the intestine*

In the beginning of the perfusion the gut exhibited some tone and rhythmic activity, but after a time it relaxed completely and showed no movements. This stage was reached earlier when the perfusion fluid contained no glucose. At the beginning the greater part of the fluid was collected from the venous cannula, but as perfusion continued the leakage

from the lumen increased and sometimes amounted to more than half of the outflowing fluid.

The effluent from perfusions with eserinated solutions caused contraction of the rectus muscle of the frog whether glucose had been added to or was absent from the perfusion fluid. Usually the stimulating effect was strongest in the early samples, decreasing after 30–60 min. of perfusion, at first quickly and then more and more slowly. The contraction of the frog rectus resulted in part from acetylcholine, in part from choline and from an unidentified substance or substances. Destruction of acetylcholine by NaOH abolished only part of the stimulating action, and the fluid then assayed on the rectus muscle against choline yielded a higher choline value than the choline equivalent obtained by acetylation of the sample. For instance, in Exp. 3 of Table 1 the perfusate collected during 1 hr. perfusion contained 4.5  $\mu$ g. of acetylcholine. After destruction of the acetylcholine by NaOH the perfusate was assayed against choline on the rectus muscle. If the alkali-resistant stimulating principle had been wholly choline the perfusate would have contained 11.9 mg., but the choline equivalent obtained by acetylation was 2.1 mg. The unaccounted part must therefore have been due to the presence in the fluid of an unidentified principle, which need not have had a stimulating action but may have sensitized the muscle to choline. The discrepancy between the choline estimates obtained by acetylation and by direct assay of the perfusate was usually greatest in samples which showed strong frothing when air was bubbled through them.

Two series of experiments were carried out, the results of which showed that glucose apparently did not influence the output of choline and acetylcholine from the intestine. Perfusions were started without glucose. After 1½–2 hr., when the output had come to a relatively steady level, 50–200 mg. of glucose (or of pyruvate) were injected into the arterial cannula or the perfusion fluid was changed over to one containing 0.1 % of glucose. This caused a powerful stimulation of the gut which continued for several minutes and then disappeared even if the perfusion was continued with glucose. The change over to glucose containing perfusion fluid did not increase the output of stimulating substances, and if their concentrations had been declining they continued to do so. The injections of glucose or pyruvate sometimes produced a slight evanescent increase, but in other experiments the opposite effect was obtained. Similar slight changes were obtained after an injection of a few c.c. of glucose-free solution.

In other experiments the intestines were perfused either with or

without glucose, perfusate was collected during 1 hr. perfusion and assayed for acetylcholine, choline and the unknown stimulating principle. The latter was expressed as choline and was obtained by subtracting the choline equivalent obtained by acetylation from the value obtained by assaying the alkali-resistant effect of the sample against choline on the frog rectus. The collection of the effluent was started 40 min. after tying in the arterial cannula. In the first experiments the stimulating effect appeared to be more pronounced when glucose was absent from the perfusion fluid. Later experiments did not confirm this result and showed that there were great individual variations. Table 1 gives the results

TABLE 1. Output of acetylcholine, etc., during one hour's perfusion from 100 cm. small intestine

Exp.	Perfusate contains	Equivalent of acetylcholine chloride in $\mu$ g.			Equivalent of choline chloride in mg. by acetylation			Unidentified substance expressed in mg. of choline chloride			Length of perfused piece in cm.
		a	b	(a+b)	a	b	(a+b)	a	b	(a+b)	
1	Glucose, eserine	0.5	1.0	1.5	0.7	0.8	1.5	0.1	0	0.1	97
2	Glucose, eserine	1.5	2.0	3.5	0.5	1.0	1.5	0.3	0.5	0.8	138
3	Glucose, eserine	0.5	4.0	4.5	0.4	1.7	2.1	3.6	6.2	9.8	103
4	Glucose, no eserine	—	—	—	0.6	1.0	1.6	1.8	1.8	3.6	75
5	No glucose, eserine	0.9	0.9	1.8	0.8	1.0	1.8	3.9	2.8	6.5	105
6	No glucose, eserine	1.8	3.7	5.5	0.7	0.7	1.4	1.1	1.5	2.6	94
7	No glucose, eserine	2.2	3.0	5.2	1.0	1.9	2.9	1.1	2.4	3.5	108
8	No glucose, no eserine	—	—	—	0.4	1.8	2.2	0.9	2.8	3.7	96

obtained from eight experiments. The perfusate collected from the venous cannula and from the lumen were assayed separately. The results for the venous perfusate are given in the columns *a*, those of the fluid from the lumen in the columns *b*. The results are calculated for 100 cm. length of intestine, the actual length of the perfused part being given in the last column.

## DISCUSSION

Increased synthesis and liberation of acetylcholine or choline is not the cause of the stimulating action of glucose on the longitudinal muscle. If that were so any increase in the concentration of acetylcholine around the muscle should have a similar effect, but acetylcholine added to the bath was unable to produce sustained contraction, and large doses given

at short intervals rendered the longitudinal muscle inexcitable. Similarly, if the acetylcholine which is continuously liberated from the intestine was allowed to accumulate by using eserine to prevent its destruction no contraction or only an evanescent one occurred on the longitudinal muscle. The circular muscle, however, contracted, a fact which may be taken as evidence that accumulation of acetylcholine took place in this condition. Direct evidence for our conclusion was provided by the perfusion experiments. Glucose, despite its stimulating effect, caused no increase in the output of choline or acetylcholine, and the continuous output of these substances was of the same order when perfusion was carried out with a solution free of glucose or containing it. Since increased synthesis could have a stimulating effect only if the substances were also liberated in increased amounts the results exclude an increase in liberation as well as in synthesis. The observations on cooled preparations appeared at first more difficult to interpret. The failure of glucose to stimulate the intestine after it had lost the ability to synthesize acetylcholine suggested a close connexion between the two mechanisms. The failure, however, could be attributed to the fact that the energy stores of the longitudinal muscle did not become depleted in a cooled preparation in which the muscle exhibited only weak rhythmic activity. If such a depletion was brought about before the cooling, glucose retained its stimulating effect.

Our results are in agreement with the concept that the normal tone and rhythmic activity are due to the inherent property of the longitudinal muscle but are greatly enhanced by and partly dependent upon the continuous release of choline and acetylcholine. Glucose stimulates the muscle by supplying the chemical energy necessary for the restoration of the normal activity and tone and by making it sensitive to the released acetylcholine and choline. Neither of these factors alone would explain all our observations. The lack of a strict parallelism between sensitivity of the longitudinal muscle to acetylcholine and its tonic and rhythmic activity shows that increased excitability is not the sole cause of the stimulating effect of glucose. The first effect is in fact an increase in excitability without change in activity or tone, since doses of glucose too small to have a stimulating effect were able nevertheless greatly to increase the sensitivity of the muscle to acetylcholine. The effect of phloridzin also stresses the role of glucose as energy source for the muscle fibre. It completely suppressed the stimulating action of glucose but did not influence the acetylcholine contraction of a preparation suspended in glucose-free Tyrode solution. The effects of atropine are best explained



if both factors are taken into account. The immediate relaxation of tone and diminution or cessation of rhythmic activity it produces when given at the height of a strong stimulating effect of glucose may result from a sudden abolition of the effect of the continuously liberated choline and acetylcholine. The reappearance of tone and activity, despite the fact that the atropine remains in contact with the gut and that added acetylcholine remains ineffective, could be explained as due to the inherent property of the muscle fibre for which the glucose supplies the necessary energy. Experiments of Neukirch & Rona [1912] on the rabbit's heart can be explained only on the assumption that glucose provides the necessary chemical energy. Glucose stimulates the perfused heart although it is inhibited by choline and acetylcholine. It is unlikely that the mechanism for the stimulating action differs fundamentally from that of the intestine.

On the other hand, some observations clearly show that under certain conditions glucose stimulates the intestine only if sufficient acetylcholine is present. On cooled preparations it was often necessary to add acetylcholine or eserine to the bath in order to demonstrate the stimulating effect of glucose or the effect could be enhanced and accelerated by this procedure. On fresh preparations suspended in such a way as to record volume changes the stimulating effect of glucose sometimes disappeared with repeated administration. In such a condition it reappeared when the glucose was given with eserine or acetylcholine, although these substances alone had no stimulating effect on the longitudinal muscle.

The increase in the response to repeated administration of glucose may be due to the fact that the energy stores in the muscles are used up gradually, or more likely to the fact that the muscle becomes gradually more sensitive to the released choline and acetylcholine. This assumption is in agreement with observations on changes in sensitivity often seen on smooth muscle preparations. A gradual increase in tone of the longitudinal muscle which we have sometimes observed on preparations suspended in glucose-containing Tyrode solution could be explained similarly.

The conception that glucose exerts a stimulating action when a muscle has been more or less depleted of its energy stores provides a plausible explanation for the lack of a strong stimulating action on the circular muscle, which, unlike the longitudinal, exhibits little spontaneous activity. The muscle may therefore retain sufficient energy reserves for a long time, even in the absence of glucose from the bath, being comparable in this respect to the longitudinal muscle of a cooled preparation.

The stimulating action of the various sugars and of pyruvate must be

explained in the same way as that of glucose, since in its presence their stimulating action was only slight. The fact that phloridzin inhibits pyruvate, although less than glucose, cannot be explained on the known action of phloridzin. The inhibition of the action of glucose can be attributed to inhibition of the first phosphorylation stage; there is no evidence that phloridzin has a similar action on the oxidation of pyruvate. Unlike the various sugars and pyruvate, lactate appears to be unable to replace glucose for the functioning of the longitudinal muscle, or it has this property only to a slight extent. Its stimulating effect on the longitudinal muscle must be regarded as a direct effect. Otherwise it would not be more pronounced when glucose is present in the suspension fluid and so often disappear when, in the absence of glucose, the muscle becomes more or less depleted of its energy stores. The strong stimulating action which lactate exerts on the circular muscle must also be regarded as a direct effect. If we compare the possible role of lactate in the metabolism of the active longitudinal muscle of the rabbit's intestine with that of the heart muscle a striking difference is evident. The heart not only readily oxidizes lactate but actually prefers it to glucose [Evans, Grande & Hsu, 1935].

There are some differences and parallelisms between the actions of the various sugars and related substances on the longitudinal muscle of the intestine and in their ability to promote synthesis of acetylcholine in brain tissue or in a perfused sympathetic ganglion where the synthesis resulted in restoration of conductivity across the ganglionic synapse. On the intestine galactose was 2-3% and mannose 25-30% as active as glucose, whereas the respective figures on the ganglion were 5 and 60%. Laevulose had no effect in restoring conductivity in the ganglion, it had a doubtful effect in accelerating synthesis of acetylcholine in brain tissue, and a slight stimulating effect on the longitudinal muscle of the intestine. Lactose and maltose did not stimulate the intestine nor did they restore conductivity in the ganglion or accelerated synthesis of acetylcholine in brain tissue. On the other hand, pyruvate, which on the intestine was many times more effective than glucose, was less than half as active on the ganglion. The greatest disagreement was observed with regard to lactate. Its activity in accelerating synthesis of acetylcholine appeared to be equal to that of pyruvate in the ganglion and brain tissue, whereas it was unable to replace glucose in the intestine. Different sugars and allied substances appear, therefore, to vary in their ability to replace glucose in different physiological processes.

According to Kahlson & MacIntosh there are definite differences in

the latencies of the restorative effects of pyruvate, glucose and galactose, that of pyruvate being shorter and that of galactose longer than that of glucose. Similar differences occurred in the latencies of the stimulating effects of these substances on the intestine suggesting identical metabolic events for the substances in both processes. Kahlson & MacIntosh have suggested that glucose may act after conversion into pyruvate and galactose after conversion into glucose.

### SUMMARY

1. Preparations of the isolated small intestine of the rabbit were suspended in Tyrode solution, with and without glucose, and the reactions of the longitudinal and circular muscle were studied and recorded separately. The following observations were made:

(a) *Acetylcholine*, *muscarine* and *eserine* stimulate both the longitudinal and circular muscle. The effect on the longitudinal muscle disappears in glucose-free solution, whereas that on the circular muscle persists and is then the sole response to be obtained.

(b) *Glucose* and *pyruvate*. In glucose-free Tyrode solution the longitudinal muscle relaxes, its rhythmic activity diminishes or disappears and acetylcholine no longer causes sustained contractions. Following repeated administration of acetylcholine at short intervals the muscle may become insensitive to it and to other stimulating substances. In this condition glucose and pyruvate greatly stimulate the muscle, and the effect is followed by a period of increased excitability of the muscle. On the circular muscle the stimulating effect of glucose and of pyruvate is small and irregular. Glucose and pyruvate added to a preparation suspended in Tyrode solution containing glucose cause slight inhibition followed by slight stimulation of the longitudinal muscle.

(c) *Laevulose* had a slight stimulating effect on the longitudinal muscle, that of *galactose* was 3-4% and that of *mannose* 25-30% that of glucose. Large doses of these sugars produced slight immediate inhibition which was best seen in the presence of glucose. *Maltose* and *lactose* had no stimulating action.

(d) *Lactate* differs in its action from glucose or pyruvate in the following ways: (1) it stimulates mainly the circular muscle, (2) this effect is not influenced by glucose, and (3) stimulation of the longitudinal muscle is more pronounced in the presence of glucose than in its absence. In this condition it renders the longitudinal muscle less sensitive to the stimulating action of glucose.

(e) *Phloridzin* inhibits the stimulating action of glucose and to a

smaller extent that of pyruvate. In the absence of glucose it has no effect on the muscle and does not influence its responses to lactate or acetylcholine.

(f) *Atropine* given during the action of glucose may cause complete relaxation of the longitudinal muscle and cessation of its rhythmic activity. The effect soon disappears even if the atropine is left in the bath and the muscle insensitive to added acetylcholine. Small doses of atropine abolish the stimulating effect of acetylcholine and of muscarine on the longitudinal as well as on the circular muscle.

2. The stimulation of the longitudinal muscle by glucose is not due to increased synthesis of acetylcholine or choline in the intestinal wall. It is due to the fact that glucose supplies the chemical energy necessary for the tonic and rhythmic activity of the muscle and makes it excitable to the acetylcholine and choline continuously released in the intestinal wall. This conclusion is based (1) on the response of the muscle to acetylcholine in the absence of glucose, (2) on the fact that glucose does not influence the release of acetylcholine and choline from the perfused gut, and (3) on the observation that a cooled preparation which has lost its property of synthesizing acetylcholine is stimulated by glucose provided its reserves of chemical energy have been depleted before the cooling. The relative inability of glucose to stimulate the circular muscle may be explained by the fact that this muscle lacks powerful spontaneous activity and therefore does not become depleted of its energy stores in the absence of glucose. Pyruvate, mannose and galactose can replace glucose in its action on the intestine and their stimulating effects must be explained in the same way as that of glucose. This does not apply to the stimulating actions of lactate which must be regarded as direct effects.

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THE DIURETIC ACTION OF ALCOHOL IN MAN<sup>1</sup>

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It is a matter of common observation that the taking of alcoholic drinks is followed by an increased output of urine. In the case of beer-drinking, this is not surprising, since large volumes are usually consumed; and in the case of some other beverages, e.g. gin, a diuretic agent is present in the drink. The observed diuresis is usually attributed to a combination of these two factors, and most writers either state categorically or imply that alcohol per se has no diuretic action. In 1932, however, from a comparison of the diuretic effects of a given volume of water, with and without alcohol, on two subjects, Murray concluded that alcohol itself was exerting a diuretic action. No alcohol estimations were made, but from observations on the composition of the urine (chloride and phosphate concentrations) and on the inhibitory action of pituitary extract, it was concluded that the mechanism of alcohol diuresis was of the same nature as that of water diuresis.

The diuretic action of alcohol has now been demonstrated on five other subjects, and its mode of action investigated by simultaneous analysis of alcohol concentration in the blood and urine.

## METHODS

To each subject the same volume of fluid was given on different occasions; it had a constant basis of cider (70%) to render it more potable and varied only in its alcohol content. Since the experiments were often of long duration and avoidance of fatigue necessary in view of other aspects of the experiment, a light breakfast was allowed (tea or coffee, toast and butter). The dose of alcohol was always given 2½-3 hr. after this, and was usually drunk in the space of 10-15 min. along with

<sup>1</sup> The work was aided in its initial stages by a grant from the Rockefeller Foundation to the Maudsley Mental Hospital.

two or three dry biscuits. The bladder was emptied prior to this, and urine samples collected at frequent intervals. The subjects used appeared to experience no difficulty in attaining complete emptying of the bladder, except occasionally when in an advanced stage of intoxication.

Blood samples were collected from a pin-prick in the bent capillary tubes described by Widmark [1922]. Immersion of the hand in hot water, and use of a sharp glass pricker, ensured a free supply of arterial blood from which 0.1–0.2 g. could be readily obtained. The capillary tube was then weighed on a torsion balance, the blood blown out into a measured 1 c.c. of water in a small vessel, and the empty tube reweighed. By immediate thorough washing, each tube could be used indefinitely; a saturated solution of oxalate was drawn through and the tube then dried by passage of an air current. The dried oxalate weighed not more than 1 mg. and was ignored in the blood weighings, but served to lengthen materially the clotting time of the blood.

The water and blood were well mixed and could be left in safety in the ice chest for analysis the following day, provided the vessels were of such a size that no appreciable air space existed above the fluid. The analysis of blood alcohol concentration was then carried out in the same way as that already described for undiluted plasma [Eggleton, 1940]; 1 c.c. of the diluted blood was used, the distillate received into  $N/100$   $K_2Cr_2O_7$  in 50 %  $H_2SO_4$  in place of  $N/20$ , and the titration carried out with  $N/200$  thiosulphate in place of  $N/40$ . With these dilutions, great attention to detail was necessary at every stage in the proceedings, and a final accuracy of 2–3 mg./100 g. was attained in place of the  $\frac{1}{2}$ –1 mg./100 g. with the stronger solutions. One source of error was undoubtedly the rubber connexions between the tube of the distilling flask and that of the receiving vessel. A variable error of 0.02–0.04 c.c.  $N/40$  thiosulphate ( $\frac{1}{2}$ –1 mg. alcohol/100 g. blood) from this source passed unnoticed in the original method, but was magnified to 0.1–0.2 c.c.  $N/200$  thiosulphate (2–5 mg. alcohol/100 g. blood) with the weaker solutions, and an average blank value of 0.15 c.c. had, therefore, to be subtracted from all titres. Analysis by both macro- and micro-methods of blood to which alcohol had been added indicated that recovery was equally complete in the two, although the variable error was greater in the micro-method.

This method was evolved as an alternative to the Widmark method, since conditions prohibited satisfactory use of the latter. There the titration, with consequent possibility of contamination with tap grease, etc., is carried out in the same flask in which the dichromate solution in concentrated sulphuric acid is kept during the distillation; the flasks, therefore, must be cleaned chemically between each estimation and then thoroughly dried. Without large numbers of the apparatus and a dust-protected room or large cupboard for

their sole use, serial blood sampling is impossible. A further disadvantage of such specialized apparatus is the dependence of analytical accuracy on its exact shape. In the dozen apparatus made for me in this country, the small cup which holds the blood was made rather deeper and less wide than that in the original, with the result that complete distillation of the alcohol required 3 hr. instead of 2 hr.

Under suitable conditions, it is likely that the Widmark method might yield more accurate results than the one described above, but in many circumstances the simplicity of the latter would make its use preferable. In any case, the errors involved are small in comparison with those likely to be encountered on the experimental side. Unless free bleeding is induced by the prick (and this is sometimes difficult in subjects with thick skins, or in those who do not readily tolerate hot water) and the sample taken quickly into the capillary tube, erratic values are obtained. If the finger is squeezed unduly, plasma may preponderate, leading to artificially high values; and if the blood is not taken quickly, alcohol evaporates from the relatively large surface exposed, and artificially low values are obtained.

## RESULTS

### *Diuresis*

*Relation to dose of alcohol.* In four subjects, two or three different doses of alcohol were given on different days, and the total urine output measured until the resultant diuresis had come to an end, usually in

TABLE 1. The relation between dose of alcohol and degree of diuresis

Subject	Weight kg.	Drink		Total urine output (2½ hr.) c.c.
		Volume c.c.	Alcohol content g.	
H, ♂	70.5	300	8	108
		300	59.5	858
C, ♂	63	300	8	114
		300	52	830
		300	74	1360
A, ♂	60.5	300	8	82
		300	60	642
S, ♀	66	200	26	710
		200	55	1320

2-2½ hr. The results are presented in Table 1. The only variable factor in the drink was the amount of alcohol, and it is seen that the degree of diuresis in all subjects varied with the dose of alcohol. Under the conditions of these experiments, i.e. a light breakfast 2-2½ hr. beforehand, all subjects are in water deficit, and respond to the small dose of alcohol (that contained in the cider) by retaining much of the fluid.

In the fifth subject, many more experiments were performed, and the results of these are shown in Fig. 1. There is a considerable scatter of the individual points around the mean, but the general trend is undoubtedly the same. Roughly, every extra 10 g. alcohol results in an extra 100 c.c.

urine excretion. In all of the experiments on this subject, the drink was 200 c.c. in volume (70 % cider) and varied only in its alcohol content. Of the possible factors responsible for the degree of variation indicated in Fig. 1, one was found to be of unexpectedly great importance, namely,

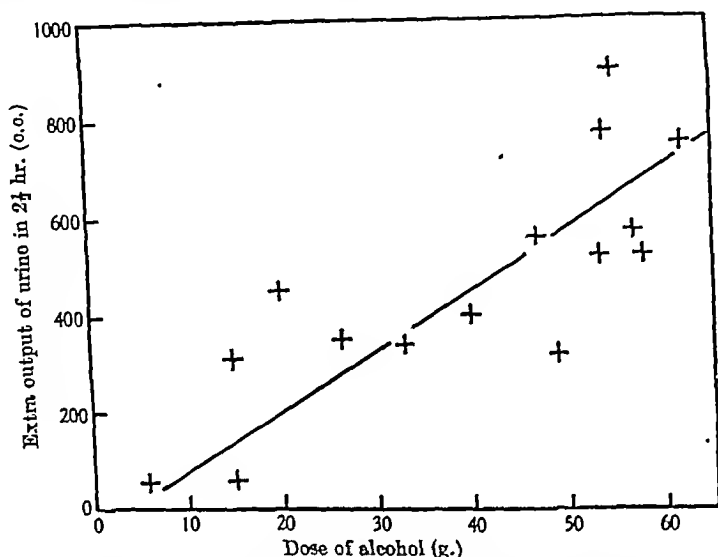


Fig. 1. The relation between dosage of alcohol and diuretic response in one subject when the volume and other constituents of the drink are kept constant.

room temperature. These experiments were carried out during the months of January to July, and although the laboratory was well warmed in winter and on the cool side of the building in regard to summer heat,

TABLE 2. The effect of external temperature on the diuretic response to alcohol in one subject

Dose of alcohol g./kg.	Room temp.	Total urine output (2½ hr.) c.c./g. alcohol/kg. body weight
0.38	Cold	1420
0.28-1.17	Normal	930 ± 105 (10)
0.62	Warm	720
0.28	"	560
0.98	"	430
1.1	Very warm	550
0.92	"	440

there was a considerable variation in temperature throughout the period. No records of this were kept, but only extreme variations noted. Experiments in which this was done are quoted in Table 2. In view of the large differences observed on these occasions, it seems likely that some of the



variation amongst experiments in the 'normal' group was also due to unnoted temperature differences. In later sets of experiments, therefore, every effort was made to reduce the variability of this factor.

*Time relation to blood-alcohol concentration.* It was hoped that further information as to the mechanism of this diuresis might be obtained from a knowledge of the relationship of the course of diuresis to changes in the

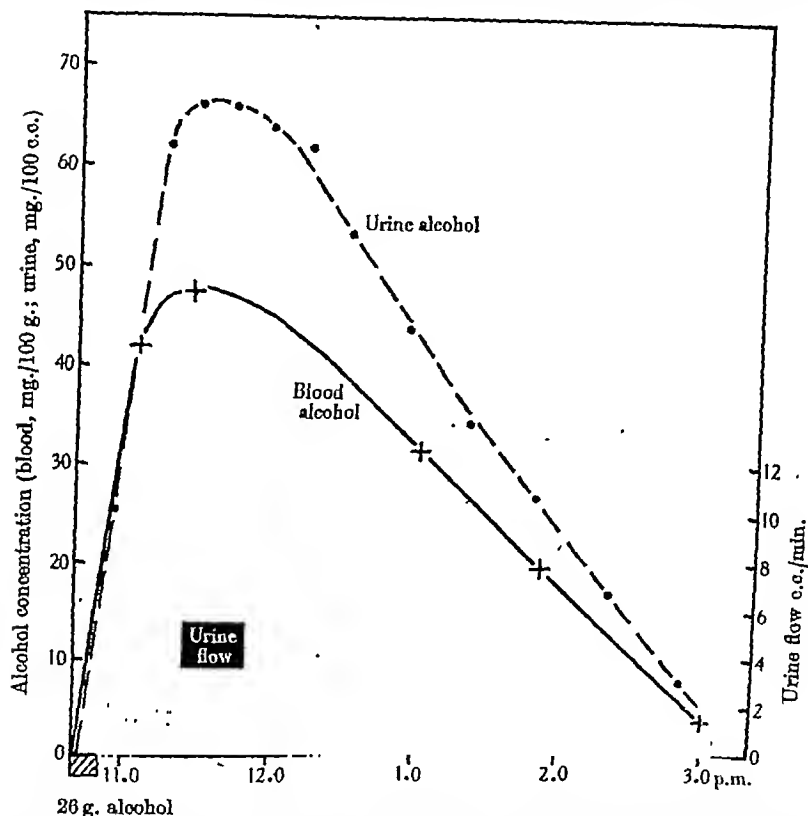


Fig. 2. The relation between the alcohol concentrations in blood and urine and the course of diuresis when absorption of the alcohol is slow. Subject S, 68 kg., 26 g., alcohol in 200 c.c. fluid taken at 10.40-10.52 a.m.

blood-alcohol concentration. The results of the experiment depicted in Fig. 2 are typical of those obtained when absorption of the alcohol is slow. The height of diuresis coincides approximately with the peak value of blood-alcohol concentration. This apparent relationship, however, is a coincidence. If absorption is faster, the blood-alcohol concentration is already decreasing when the height of the diuresis is reached. Such a case is shown in Fig. 3.

The time relationship of diuresis to blood-alcohol concentration suggests that this relationship may be of hormonal nature, as suggested

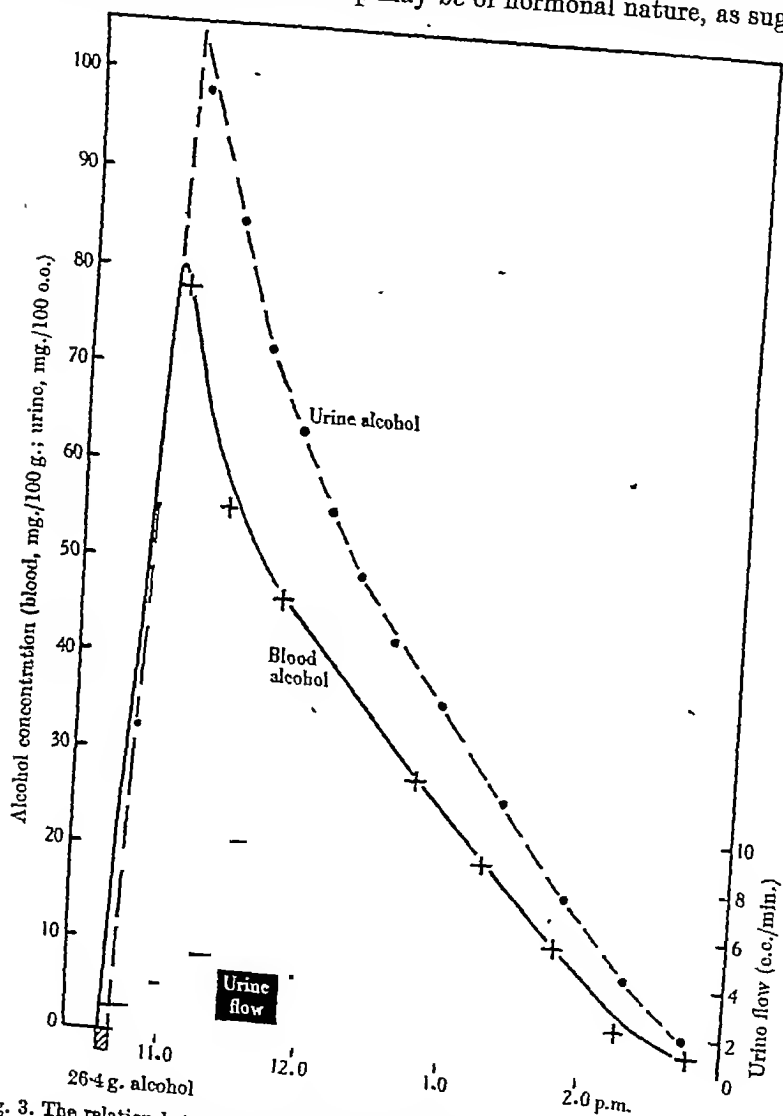


Fig. 3. The relation between alcohol concentrations in blood and urine and the course of diuresis when absorption is rapid. Subject E, 52.5 kg., 26.4 g. alcohol in 200 c.c. taken at 10.35-10.40 a.m.

by Murray [1932]. Her results have been amply confirmed; post-pituitary extract will completely inhibit alcohol diuresis, although this form of

diuresis appears to be somewhat more resistant to its action than is a water diuresis. On the first occasion, 1 unit was injected 15 min. before the alcohol was taken. The onset of diuresis was delayed until  $1\frac{1}{2}$  hr. after the alcohol had been taken, and then amounted to 450 c.c. instead of the

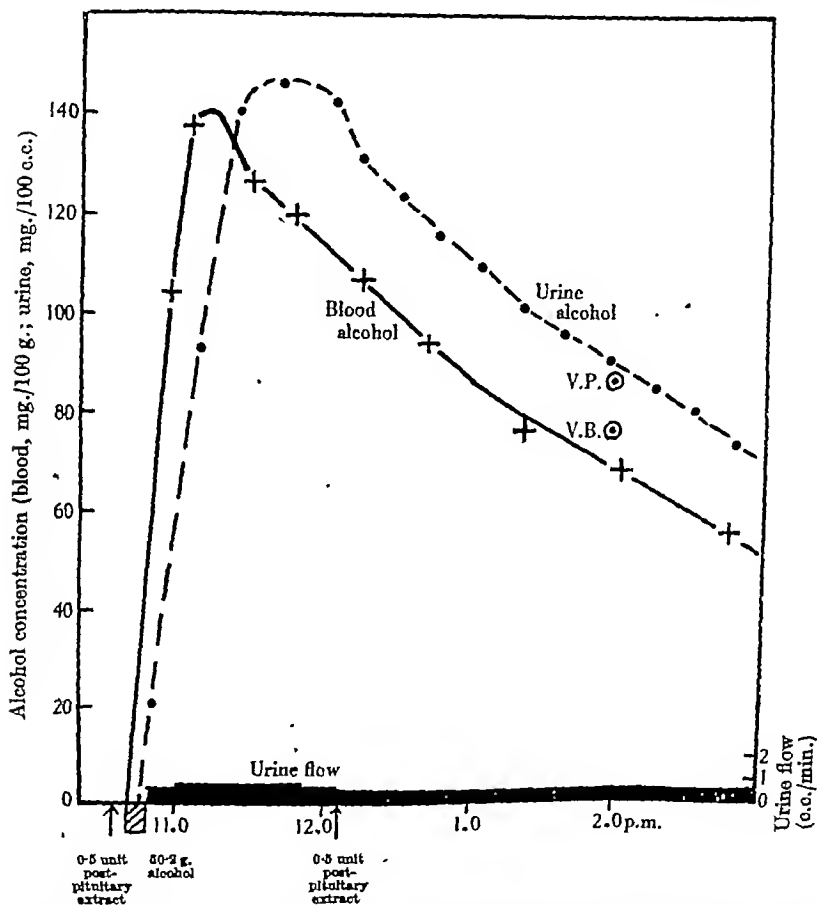


Fig. 4. The effect of post-pituitary extract in suppressing the diuretic response to alcohol. Subject E, 52.5 kg. V.P. and V.B.=alcohol concentration (mg./100 c.c.) in venous plasma and blood respectively.

600–800 c.c. expected after a dose of 50 g. alcohol. In a second case, 0.5 unit was injected 5 min. before a dose of 50 g. alcohol and another 0.5 unit  $1\frac{1}{2}$  hr. later (Fig. 4). In a third case, 1 unit was given 3 min. before 53.5 g. alcohol and another unit  $1\frac{1}{2}$  hr. later. In both of these experiments diuresis was completely absent (see Fig. 4). These results, taken in conjunction with the fact that the normal alcohol diuresis shows

the same lag in onset as a water diuresis is very suggestive that the same mechanism is responsible for both.

If this is the case, the question still remains as to whether the pituitary gland is inhibited directly, or by way of its nervous control in the hypothalamus. The latter would seem more probable in view of the depressant

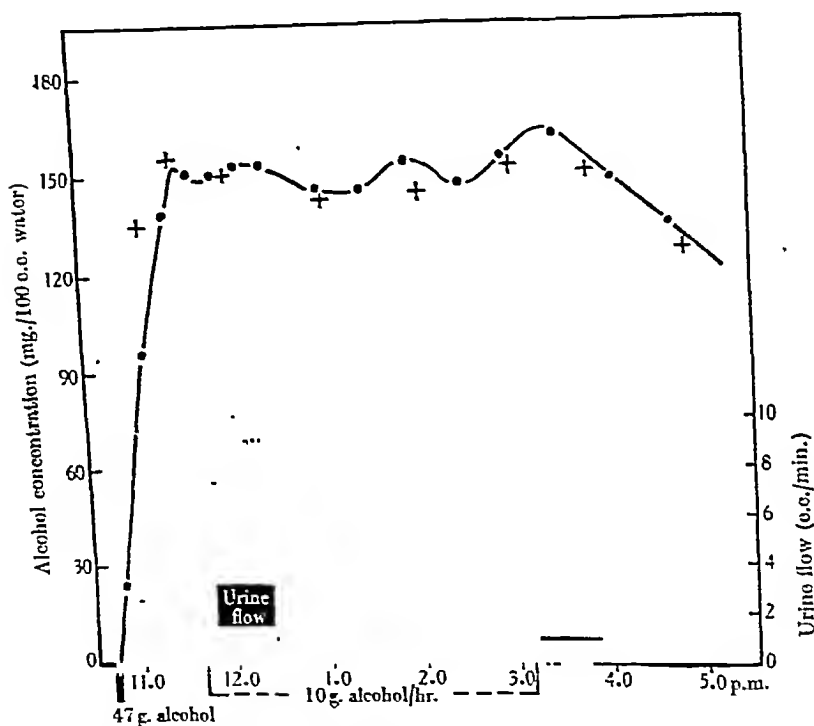


Fig. 5. The effect of a steady concentration of blood-alcohol in failing to maintain diuresis. Subject E, 53 kg., 47 g. alcohol in 200 c.c. taken at 10.40-10.50 a.m. From 11.40 a.m. to 3.10 p.m. 38.3 g. in 233 c.c. taken in 20 c.c. doses every 20 min. ●—● = urine alcohol; — = blood alcohol (concentration in mg./100 c.c. water).

action of alcohol on other parts of the central nervous system, but there are some difficulties which prevent a ready acceptance of this theory. Concerning the action of alcohol on cortical function, as reflected in sensori-motor and other tests, it has been shown that although the absolute concentration of alcohol is a potent factor in the depressant action, the rate and direction of its change may be equally important: at any given concentration, greater effect is produced if it is increasing than if it is decreasing, and this effect also varies directly with the rate of



diuresis. When this change ceases, even although the absolute concentration is maintained, the diuresis ceases just as abruptly as if only a single dose of alcohol had been given. Such a result is shown in Fig. 5. The diuresis following a dose of 47 g. is of average size (700 c.c.) and duration ( $2\frac{1}{2}$  hr.), although the blood-alcohol concentration was maintained at 105–115 mg./100 g. (140–150 mg./100 g. water) for 4 hr. The cessation of diuresis cannot be attributed to dehydration of the body because a second single dose of alcohol given after the diuretic response to the first has died away, will initiate a second period of diuresis. Such a result is shown in Fig. 6. The second response is not so large as the first, 210 c.c. in place of 410 c.c., but antagonizing factors were many: the body was in water deficit from the first diuresis, the room temperature was unusually high (noted as a very warm day), and food had been taken, withdrawing fluid into the stomach and intestines. In spite of all these dehydrating factors, the second increase in blood-alcohol concentration initiated a diuresis.

*Effect of duration of increasing blood-alcohol concentration.* The effective action of an increasing blood-alcohol concentration is thus common to both cortical function and diuretic response. If the latter is initiated through a nervous mechanism, it might be expected that it also would respond to *rate of increase* in blood-alcohol concentration in the same manner as does the cortex, and that the two types of response would vary in the same direction. Cortical function is most disturbed by a fast rate of increase in alcohol concentration, but it became clear during the course of these experiments that no such direct relationship existed in the case of the diuretic response.

Comparison of Figs. 2 and 3 suggests that the duration of the stimulus is a more important factor than the actual rate of increase or absolute concentration of alcohol. In subject S (Fig. 2) a diuresis of 710 c.c. resulted from a blood-alcohol concentration of only 48 mg./100 g. reached in 50–60 min., whereas subject E (Fig. 3) gave a response of only 440 c.c. to a blood-alcohol concentration of 80 mg./100 g. reached in 20–30 min. Thus the larger diuresis accompanied the slower rate of increase in blood-alcohol concentration and vice versa. The variation in diuretic response of five subjects listed in Table 1 and Fig. 1 tended in the same direction. This variation can be more easily appreciated from the figures in Table 3, where the diuresis is expressed in terms of a standard dose of alcohol, 1 g./kg. body weight. It would seem that the mechanism responsible for this diuresis is more than twice as sensitive in some individuals as in others, and that this variation may be partially due to variations in the duration of the

TABLE 3. A comparison of the degree of diuresis in different individuals to the same dose of alcohol

Subject	Total urine output (2½ hr.) c.c./g. alcohol/kg. body weight
S, ♀	1690 ± 110 (2)
H, ♂	1035 ± 85 (2)
C, ♂	1020 ± 80 (2)
E, ♀	835 ± 90 (16)
A, ♂	635 ± 15 (2)

increase in blood-alcohol concentration, for subject S was on all occasions the slowest absorber and subjects A and E the fastest.

The general impression gained from experiments on these five subjects was reinforced by results from further experiments on a second series of seven other subjects. Blood-alcohol determinations were no longer feasible owing to enemy action, but experience has shown that the course, though not the absolute value, of blood alcohol concentration can be fairly accurately judged from tests of cortical function [Eggleton, 1941]. The degree of upset of the nervous system reflected in these tests is greater if the blood-alcohol concentration increases more rapidly, greatest at the peak value, and decreases rapidly with decrease in alcohol concentration. The group of seven subjects, therefore, became well practised on the dotting machine, and a comparison was then made of

TABLE 4. Relative effects of the duration and intensity of alcohol action on the diuretic response

Exp.	Subject	Weight kg.	Dotting machine performance maximum error		Total urine output in 2½ hr.
			% decrease in correct hits	Time after beginning of drink min.	
1	R. A. B. ♂	90	19.5	26	240
2	D. R. W. ♂	54	17.5	37	445
3*	D. I. H. ♀	55	>36	<32	520
4	P. L. L. ♂	66	10 8	67 107	530
5	D. R. W. ♂	54	18 22	40 107	680
6	D. F. R. ♂	58	29 21	16 34	720
7	J. D. ♀	64	33 30	15 30	930
8	J. D. ♀	64	24 12	40 100	1080

A dose of 45 g. alcohol in 250 c.c. was given to all subjects.

\* Owing to a misunderstanding, the first test was not made until the subject was already beginning to recover, subjectively.

the effect of a standard dose of alcohol (45 g. in 250 c.c., water only) on their dotting performance and diuretic response. The results are presented in Table 4, in ascending order of diuretic response. Apart from the first subject listed, this response is unconnected with body weight. The general trend of the results supports the impression gained from the earlier subjects, that the duration of alcohol action, rather than a short, swift effect, is the potent factor in determining the extent of the diuresis. The occurrence of more than one peak value, due no doubt to irregular emptying of the stomach, indicates that the nervous system was subjected to more than one period of increasing blood-alcohol concentration.

*Cause of individual variation in diuretic response.* A closer survey of these results suggests that some individual personal factor may be also partially responsible for the large variation in diuresis observed. A comparison of Exps. 5 and 8, for example, shows a wide variation in diuretic response in two individuals (680 and 1080 c.c. respectively) with approximately the same rate of absorption and degree of nervous upset. The possibility that this degree of variation might be due to individual variation in sensitivity of the pituitary mechanism, if this hypothesis of the diuretic action be accepted, is an obvious one. An attempt was next made, therefore, to see whether such a variation might be responsible. In a further group of subjects, the diuretic response to both alcohol and water was studied. Both substances were given roughly in proportion to the body surface, i.e. to the weight<sup>1</sup>: a 60 kg. man receiving 38.5 g. alcohol in 200 c.c. (water only) and 1 l. of water. A wide range of response was encountered, both in dotting performance and in the two types of diuresis. The percentage decrease in correct hits in the dotting test varied from 1 or 2 % to over 40 %, and individual variation in both alcohol and water diuresis was nearly 100 %. The degree of upset of the nervous system was assessed in arbitrary units, equal weight being given to intensity and duration; thus, one unit represented 10 % decrease in correct hits in the dotting test for 10 min., or 5 % for 20 min., etc. The scores ranged from 0 to 40, but no correlation was observed between this value and the diuretic response to alcohol. Nor was any correlation of statistical significance, either positive or negative, found between water and alcohol diuresis. The complexity of factors concerned masked any such simple correlation, and the only relationship which could be established is that indicated in Table 5.

Initially, there were fifteen subjects, but three felt ill as a result of the alcohol, and the resulting autonomic disturbance stopped the renal flow; in one the water diuresis was prolonged over many hours, presumably due to delayed emptying of the stomach; and in



TABLE 5. Effect of duration of alcohol action on the relation between water and alcohol diuresis

Exp.	Subject	Peak time* of alcohol absorption min.	Diuresis (arbitrary units)†		
			Alcohol	Water	Ratio water/alcohol
1	R. E. M. ♂	15-20	22	49	2.2
2	D. M. E. ♂	25	21.5	43	2.0
3	C. F. R. ♂	25	19	32	1.7
			21 ± 1	41 ± 5	
4	S. W. S. ♂	25 and 55	19.5	33	1.7
5	J. D. ♀	30 and 50	23.5	38.5	1.65
6	J. P. ♀	45	28.5	43.5	1.5
7	M. H. ♀	45	23	32	1.4
8	R. C. ♀	50	29	35.5	1.2
9	R. J. ♀	20, 60 and 120	21	27	1.3
10	P. L. L. ♂	80 and 135	28	30.5	1.1
			24.5 ± 1.5	34 ± 2	

\* Assessed from time of worst performance on dotting machine.

† Volume excreted in relation to body weight: 1000 c.c. in 60 kg. man = 30 units. Volumes imbibed: water 30; alcohol solution 6.

another, this failure to empty the stomach occurred after alcohol, so that neither diuresis nor upset of the central nervous system was observed. This case is discussed more fully later. In all subjects, the previous meal had been omitted, either breakfast or lunch; the body was in approximate water balance, since two glasses of water were taken 2-2½ hr. before each experiment; and the alcohol and water, or water and alcohol, experiments were performed on successive days at the same time of day. By alternating the order in which the two experiments were done, the effect of changes in temperature were largely annulled. In the second part of Table 5 two values should be increased and two decreased, owing to this factor. In the first part of the Table, the lowest ratio, 1.7, should be increased.

The results have been arranged approximately in order of increasing duration of alcohol action, and subdivided into a group of three rapid absorbers and the remaining seven slower ones. The alcohol diuresis is slightly smaller in the former group than in the latter, but the difference is barely significant. The reverse is the case in respect of water diuresis, but again there is no significant difference. This tendency to a negative correlation between alcohol and water diureses might be expected if the rate of emptying of the stomach was a constant factor in any individual; rapid absorption of water is likely to produce a greater diuresis than does slow absorption, whereas rapid absorption of alcohol, with short duration of its action, appears to produce a smaller diuresis than does slow absorption.

If rapidity of absorption, both of alcohol and of water, was the only factor concerned in the diuretic response, a more orderly arrangement of increasing alcohol diuresis and decreasing water diuresis in relation to the duration of alcohol action (as indicative of the natural rate of emptying

of the stomach) might have been expected. If some further individual factor is concerned, conditioning the magnitude of the diuretic response in both cases, its effect might become apparent if the ratio of the two diureses were considered. These figures are shown in the last column of the table, and do in fact show a much more orderly sequence. A comparison of subjects 6 and 7, in whom this ratio was approximately the same, will illustrate the point in question. Both absorbed alcohol at the same rate and both showed only a slight disturbance of cortical function, but in subject 6 the diuresis was considerably greater than in subject 7. The fact that subject 6 showed also a much larger diuretic response to water (although the two water diuresis experiments were done on the same day) suggests that the mechanism concerned in both responses was more sensitive than in subject 7.

The variation in ratio, ranging from 2.2 to 1.1 in different individuals, is an expression of the opposite effects of the rate of absorption on alcohol diuresis on the one hand and water diuresis on the other. The orderly nature of the change, however, is strongly suggestive of the presence of another factor, tentatively referred to as 'sensitivity of the pituitary mechanism', which varies in different individuals. With the evidence available, this can be no more than a suggestion, and must await confirmation or the reverse from experiments under much more carefully controlled conditions.

*Relative importance of the different factors concerned.* In view of this result, the clearest evidence of relationship between duration and intensity of alcohol action on the diuretic response should be obtainable by varying these factors in experiments on one individual, in whom the 'sensitivity of the pituitary mechanism' may be accepted as approximately constant. Comparisons of some earlier experiments on one subject (E), in which blood-alcohol concentration was estimated directly, have been grouped in Table 6. In the first couple it is seen that long duration of stimulus (obtained by sipping the drink over a long time), and lower absolute concentration of alcohol, produce less depression of the cortex but greater diuresis than a short but more intense stimulus. In the second couple, the same absolute value of blood-alcohol concentration was reached, but again the longer-lasting stimulus produced a smaller cortical and larger diuretic effect than the shorter stimulus.

A modification of these experiments has been repeated on the same subject, without analysis of the blood-alcohol concentration. The same dose of alcohol was given on several occasions under identical conditions as far as possible, but with varying rates of absorption. The results

TABLE 6. Effect of duration and intensity factors in the action of alcohol on the central nervous system and on the diuretic response

Dose of alcohol g.	Blood-alcohol concentration		C.N.S. effect % increase in		Diuresis c.c./2½ hr.
	Peak value mg./100 g.	Time taken to reach peak min.	Typing time errors	Distraction machine errors*	
40	135	20	—	70	480
52	118	90	—	20	540
56	155	45	90	—	710
62	162	72	45	—	965

All experiments performed on the same subject (52 kg.), and the volume of drink kept constant (200 c.o.).

\* Details of tests given elsewhere [Eggleton, 1941].

tabulated below (Table 7) show unmistakably that the degree of alcohol diuresis is connected with the duration of the rising blood-alcohol concentration and not with the intensity of its effect on the higher nervous system. Although the general trend of these results is so definite, their

TABLE 7. Effect of duration of alcohol action on the central nervous system and on the diuretic response

Peak time* of alcohol absorption min.	Dotting machine total time errors†	Diuresis c.c./2½ hr.
10	11	455
10 and 35	38	490
5 and 50	21	630
90	0	670
90	0	770

The same dose of alcohol (35 g. in 180 c.o.) taken in all experiments by the same subject (52 kg.).

\* Assessed from time of worst performance on dotting machine in Exps. 1 to 3, and from duration of drinking time in 4 and 5.

† 1 unit = 10% decrease in performance for 10 min., 5% decrease for 20 min., etc.

regularity is not as great as might have been expected. Certain interfering factors, though uncontrollable, were recognized at the time. The third value, 630 c.c., was obtained on a 'very cold' day in contrast with the remaining values all obtained on 'fairly cold' days, and was, therefore, unduly large. The next value, 670 c.c., probably represented an absorption time of about 70 min. in place of the expected 90 min., owing to a failure of the stomach to empty during the early period of drinking. Although no signs of central nervous system disturbance could be detected by means of the dotting machine, either in this or in the last experiment, euphoria was noticed in both; it was first recorded 20 min. after the beginning of the drink in the last experiment, but not until 40 min. after in the previous one. It would seem that more accurately

quantitative results cannot be obtained on man unless the alcohol be given by duodenal tube or intravenously, and the experiments carried out in a room at constant temperature.

Clearly, there must be some limiting rate of increase in blood-alcohol concentration to which the diuretic mechanism is no longer responsive, but under the conditions employed in these experiments it was not deliberately reached. In one subject mentioned in the previous section, this state of affairs was encountered. Neither diuresis nor any effect on the central nervous system occurred after the usual dose of alcohol. An attempt was made subsequently to hasten emptying of the stomach by giving magnesia tablets with the drink, on the advice of a friend given from empirical observation of its effect. A small diuretic response was obtained (14 in arbitrary units), but results on the dotting machine indicated a very erratic behaviour of the pylorus, and the result has not been included in Table 5. His water diuresis figure was 27, one of the lowest observed.

The action of magnesia was again tested in the first experiment quoted in Table 7. The whole drink (35 g. alcohol in 180 c.c.) was taken in 5 min. with two magnesia tablets and the usual two dry biscuits. The stomach began to empty rapidly, as evidenced by a dotting score of 28% decrease in correct hits 10 min. after the beginning of the drink. The nervous system then made a smooth recovery and no further upset was observed during the 2½ hr. of experiment. The total time-errors score was very much smaller than would have been the case if any large fraction of the dose had left the stomach at this early stage, and the results suggest that the remainder left so slowly that it had no further effect either on the nervous system or in promoting diuresis. It seems likely that the same phenomenon was responsible for the small diuresis of subject D. R. W. in Exp. 2 of Table 4. The dose given produced a much larger effect both on the nervous system and on the diuretic response in a subsequent experiment (5).

The actual rate of increase in blood-alcohol concentration responsible for the larger diureses shown in Table 7 can be calculated approximately. In the 90 min. of absorption at least one-third of the 35 g. ingested would have been metabolized (the average rate of metabolism in this subject was 8-10 g./hr.), and the remaining 22 g. be distributed in about 36 kg. of tissue (body weight 52 kg.  $\times$  0.7), yielding a blood-alcohol concentration of ca. 60 mg./100 g. Thus the rate of increase in concentration during the 90 min. would be ca. 0.7 mg./100 g./min.

## DISCUSSION AND CONCLUSIONS

The evidence presented indicates that, under comparable conditions, the diuresis initiated by alcohol in any individual varies directly with the quantity taken. This relationship is disturbed (*a*) by variations in external temperature, due presumably to greater loss of fluid in sweat under hot conditions, and (*b*) by variations in the rate of absorption of alcohol. If this be slowed in the naturally fast absorber, a larger diuresis results from the same dose of alcohol.

When the response of different individuals to the same dose of alcohol (given in relation to body weight or body surface) is compared, a large variation in diuretic response is encountered. This variation appears to be due primarily to differing rates of absorption, the naturally slow absorber (due presumably to slow emptying of the stomach) responding with a larger diuresis than the naturally fast absorber. This relationship, however, is not consistently observed when the responses of groups of individuals are compared, but is obscured by some further individual variable factor.

The evidence presented as to the nature of this second variable factor is not conclusive, but suggests that it may be linked with individual variation in diuretic response to water. In the first place, an alcohol diuresis can be inhibited by post-pituitary extract in the same order of dose, as can a water diuresis, and this may be accepted as presumptive evidence that the same mechanism is concerned in the initiation of the two types of diuresis. Secondly, the naturally rapid absorber appears to give a larger diuretic response to water than does the naturally slow absorber. When due allowance is made for this opposing combination of effects, however, i.e. large water diuresis and small alcohol diuresis with rapid absorption, a further complementary relationship between the two is revealed, indicative of a natural variation in degree of diuretic response to both stimuli, water and alcohol. This is referred to tentatively as a 'natural variation in sensitivity of the pituitary mechanism'.

The further question as to whether alcohol diuresis is produced by the action of this substance on hypothalamic nerve centres or directly on the pituitary body must remain an open one at present. If the nerve centres are concerned, they differ markedly from the cortex in being more sensitive to duration of increasing blood-alcohol concentration than to its rate of increase.

#### *The relation of alcohol concentration in blood to that in urine*

It is generally recognized that alcohol concentration in the urine provides a rough guide to that in the blood, though little quantitative work has been done on this relationship since the classic research of Miles [1922]. He concluded that: 'The alcohol concentration in venous blood and in urine is not identical and does not run parallel in the first two hours after ingestion...still the urine-alcohol curve is very useful for comparison with the time relations of the objective measurements of alcohol effect on the central nervous system.' No micro-method for blood analysis was available at that time, nor was the macro-method in

use so accurate as that now available. Under modern conditions of analysis, it is clear from the data already presented (Figs. 2-5) that an accurate reflexion of changes in blood-alcohol concentration can be obtained from analysis of urine samples, provided the changes involved are not too rapid.

Frequent urine sampling is essential, especially when the rate of flow is changing rapidly. Thus, in Fig. 2 the first urine sample was secreted mainly in the second half of the collection period, and the analytical point should, therefore, be moved to the right of the mid-point of this period. Similarly, the sixth sample was secreted mainly in the first half of the sampling period, and its analytical point should be shifted to the left. The actual concentration in the urine is independent of the rate of urine flow as is well known, and the relationship between blood and urine-alcohol concentrations shown in Fig. 4 where diuresis was completely inhibited by pituitrin, is different from that shown in other figures only in respect of the lag of urine-alcohol concentration during the period of rapid change. This is undoubtedly an artefact, due to the small volume of each sample (10-15 c.c.), of which an appreciable proportion must represent a 'wash-out' of the previous sample. During later stages, when alcohol concentration is changing relatively slowly, such frequent sampling is no longer necessary and the effect of this error, therefore, is much less pronounced.

In the slowly changing curves of Fig. 2, the urine-alcohol concentration follows closely the blood-alcohol concentration, at a value 30-35 % higher, except in the early stages of absorption. With swifter absorption, the urine curve lags appreciably behind the blood-alcohol curve on its upward course, and the same lag is often observed when rapid changes occur on the down curve. It seems probable that the explanation given above accounts also for these discrepancies. The rapid changes of concentration in both directions occur only in the rapid absorber, and therefore at a time when urine samples are still small in volume owing to the lag in onset of diuresis. In view of the fact that the urine-alcohol concentration is independent of diuresis, the probability is high that free diffusion of this substance occurs between tubule fluid, renal cells, tissue fluid and blood throughout the substance of the kidney.

In all of the figures it can be seen that the urine-alcohol concentration is 30-35 % higher than blood-alcohol concentration except during the periods of rapid change. The question arises as to whether this difference is compatible with the assumption that the alcohol in urine is in equilibrium with that in the plasma. In the experiment depicted in Fig. 4, a

venous sample was taken at one point, enabling analysis by the more accurate macro-method of both blood and plasma. This was repeated in a second experiment, and the results of the two in conjunction with those of the micro-analysis of arterial blood and macro-analysis of urine are presented in Table 8. The ratio of plasma alcohol/blood alcohol, 1.13-1.16,

TABLE 8. The relation of alcohol concentration in urine to that in blood

Alcohol concentration in arterial blood mg./100 g.	Alcohol concentration in venous		Alcohol concentration in urine mg./100 c.c.
	Blood mg./100 c.c.	Plasma mg./100 c.c.	
71	77	87	91.5
66	69.5	80.5	87.5

Alcohol concentration (mg./100 c.c.) in water of			
Arterial blood	Venous blood	Venous plasma	Urine
94	96.5	97	95.5
87	87	89.5	91

agrees with results obtained by Miles. In the second part of the table, the analytical figures have been recalculated in terms of alcohol concentration in the water of each fluid, using the average figures of 80 % in blood, 90 % in plasma and 96 % in urine. The difference between venous and arterial blood is satisfactorily accounted for by the fact that the former was measured by volume and the latter by weight (blood specific gravity, 1.06).

Within the limits of experimental error, including possible deviations from the average in the water content of the various fluids, it is seen that alcohol concentration is the same in the water of blood, plasma and urine. This conclusion is reinforced by the results shown in Fig. 5, in which all analytical figures are expressed in terms of alcohol concentration in the water of blood and urine. It would, therefore, seem justifiable to conclude that urine-alcohol concentration represents alcohol concentration in the tissue fluids, and is practically the same as that in the water of plasma except when rapid changes in concentration are occurring.

#### SUMMARY

1. A method is described for the estimation of alcohol in blood obtained by pin prick (0.1-0.2 g.). No specialized apparatus is required. Recovery is quantitative, with a variable error of 2-3 mg./100 g.

2. The diuresis following an alcoholic drink is roughly proportional to the amount of alcohol present, when volume and other constituents

are maintained constant (Table 1, Fig. 1). Variations in external temperature may seriously affect this relationship (Table 2).

3. Its onset is delayed for 20-30 min. after the drink has been taken, and the height of diuresis is unrelated in time to the peak of blood-alcohol concentration (Figs. 2, 3).

4. The diuresis can be completely inhibited by post-pituitary extract (Fig. 4).

5. It is initiated by the *increase* in blood-alcohol concentration and fails to be maintained if this concentration is kept steady, even at a high level (Figs. 5, 6).

6. The degree of diuresis resulting from a given dose of alcohol varies widely in different subjects (Table 3).

7. This individual variation is dependent on the natural rate of absorption of alcohol (Table 4) and possibly on a variation in the sensitivity of the pituitary mechanism, as evidenced by variation in the diuretic response to water (Table 5). This variation also appears to be due, in part, to varying rates of absorption of water.

8. The diuretic response to alcohol differs markedly in one respect from that of the cerebral cortex. The latter is most affected by the *rate* of increase in blood-alcohol concentration: the greater this rate of increase, the greater the disturbance of function at any absolute concentration. The diuretic response, on the other hand, is dependent mainly on the *duration* of increasing blood-alcohol concentration and not on the rate of increase (Tables 6, 7). The naturally slow absorber, therefore, tends to give a larger diuretic response than the rapid absorber.

9. When equilibrium has been established in the body, alcohol concentration in the urine remains 30-35 % higher than that in the blood. Comparison of these values with those of arterial blood, and venous blood and plasma indicate that the alcohol in urine is in equilibrium with that in the water of plasma (Figs. 4, 5, Table 8).

The major part of this work was performed at Cardiff, and I wish to express my gratitude to Prof. Graham Brown for his generous offer of hospitality at the outbreak of war.

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# EFFECTS OF TRIMETHYLAMINE ON GROWTH AND SEXUAL DEVELOPMENT IN THE RAT

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THE tertiary alkylamine, trimethylamine ( $\text{NC}_3\text{H}_9$ ), and the quaternary alkylamine, trimethylamineoxide ( $\text{NC}_3\text{H}_9=\text{O}$ ), are derived mainly from the alkanolamines, choline (betaine), whose sources are the lecithines, proteins (oxyaminoacids), of the body and also of the food. The enzyme cholinesterase also rapidly and constantly hydrolyses acetylcholine, ubiquitous in every nervous and muscular activity, to the almost inert derivative choline. Choline (betaine) readily splits trimethylamine and its oxide off under hydrolytic and oxidative agencies—under the influence of many bacteria, and also within the living cells of higher plants and animals. The major part of these metabolites is deaminized and re-synthesized in the liver of mammals (via  $\text{NH}_3$  to urea); a very much smaller fraction is excreted in the urine as the oxide, and only a minute quantity as free trimethylamine [Guggenheim, 1940]. Trimethylamine is known to accumulate in the sex organs of many plants and animals [Czapek, 1925]. Havas [1938] reported recently that *Rhodeus amarus*, *Rana esculenta* and *Triton cristatus*, when placed in an aquarium containing  $1:10^4$  to  $1:10^6$  trimethylamine, 3-4 months after the cessation of the mating season, soon begin to show their 'nuptial colours' and exhibit quite unseasonable mating behaviour. Zweifel [1877, 1881] pointed out the existence of trimethylamine in vaginitis emphysematosa or colpohyperplasia, and further suggested that its presence in the vesicles of the vagina might be responsible for this pathological condition. He also expressed the suspicion that trimethylamine was always present, in varying amounts, in the vagina of the healthy woman. Michin [1903] also called attention to the presence of trimethylamine in the vaginal secretions of women of child-bearing age and its complete absence after the climacteric.

Only a few of the more pertinent studies of choline and its derivatives can be referred to here. Immediately preceding, and during the first 2-3 days of menstruation, the choline content of blood, sweat, tears and milk is at its highest level. The sweat during menstruation contains about 80-100 times as much choline as that during the intermenstruum according to Sieburg & Patzschke [1923], who studied the effects of sweat on the movements of the small intestines of rabbits and of frog's hearts after transforming the choline into acetylcholine. Klaus [1925, 1927] found 318 mg./l. choline in the sweat of menstruating women and only 6.5 mg./l. during the intermenstruum by direct chemical determinations. To a large extent the choline in the sweat is transformed into trimethylamine by bacterial activity on the surface of the skin, thus accounting for the characteristic odour frequently encountered during this period (1/500 mg. of trimethylamine is easily distinguishable by smell). It may be of interest to observe here that the plant *Chenopodium vulvaria* L. gives off appreciable amounts of trimethylamine. The similarity of the odour of this plant to that of vaginal secretions and sweat of menstruous women was fully recognized in medieval times, if not earlier. Certainly it was known to, and formed the subject of, speculation to the old botanists and herbalists [Dalechamps, 1587].

Choline is also excreted in large amounts in the menstrual blood where, on its way out of the body, it is partly converted into trimethylamine [Klaus, 1925, 1927]. Recent studies indicate that menstrual blood is strongly toxic, and that it has some progesterone-stimulating effects in mature rats [Watkins Smith & Smith, 1940]. Lanczos [1930] demonstrated the irreversible loss of excitability of nerve-muscle preparations of frogs when held by menstruating women for 3-15 min. This phenomenon was not observed during the intermenstruum. Paralysing effects of choline chloride solutions on such preparations are only demonstrable in concentrations far exceeding any choline concentration in the sweat. Trimethylamine solutions, however, are toxic for these preparations in low concentrations. Lanczos concluded that Klaus's hypothesis to the effect that trimethylamine (derived from choline, which in turn is excreted in the sweat) might possibly be considered to be the active agent of the much discussed 'menotoxin', was not in contradiction with quantitative measurements of excitability and irritability of frog's nerve-muscle preparations.

Phytopharmacological studies—using the growth of *Lupinus alba* and *Vicia faba* as indicators—point to the existence in blood serum, blood cells, sweat, milk, tears, urine and other secretions of menstruating

women, of a poison (menotoxin) closely related to oxysterol [Macht & Davis, 1934]. These results have not been confirmed by other investigators using similar methods [Mandelstamm, Tschalkowski & Bouderenko, 1933]. The chemical methods for determining trimethylamine and its oxide in the organs, body fluids and excretions have been progressively refined [Langley, 1929]. The daily excretion of free trimethylamine in the urine of healthy men on a mixed diet is 4.22 mg., of trimethylamine-oxide 66.46 mg.

This paper is a study of the effects of injections of trimethylamine upon the growth and the development of sexual maturity of white rats (Wistar strain).

#### METHOD

Litters of rats (litter size between six and fourteen) were divided equally into control and test groups. Subcutaneous or intraperitoneal injections were started between 11 and 15 days of age (average 13 days) and were continued daily (every second day with the highest dosage) for 18–24 days (average 21 days). A 33% solution of trimethylamine in water purchased from the Eastman Kodak Company, Rochester, N.Y., was used. A dilution of one part of trimethylamine in 500–250 parts of Ringer solution (*pH* of this dilution: 10.42–10.55) is locally non-irritant, while more concentrated solutions are irritant. The local irritant effects of various concentrations were studied in preliminary experiments. Apart from outright local ulcers, weaker solutions lead to subcutaneous oedema, which is later replaced by brawny induration, so that in a few days the stiffness and fixation of the pelt seriously interferes with further injections. To avoid the irritant effects of higher dosages (100–200 mg./kg.) it was found necessary to inject portions of the dose at four different places at the same time. In most experiments control animals received equal amounts of pure Ringer solution. All animals were weighed daily. An interesting observation was that growth of hair over sites of injection of trimethylamine in higher concentration was slightly impaired—there were areas of thinning, and in some places the hair was shorter and wanting in sheen notwithstanding the fact that the pelt was loose and in the absence of other signs of local irritation. 1.5% choline injected subcutaneously into white rats leads to the falling out of the hairs at the place of injection [Exner & Zdarek, 1905]. At the conclusion of the experiment, when the males were 30–40 days and the females 40–50 days of age, all animals were killed. The glands of internal secretion as well as the liver, spleen and kidneys were preserved in Bouin's fluid for future histological investigations.

## RESULTS

In Table 1 the average weights in grams of six subgroups of animals are computed at the ages of 10, 20, 30 and 40 days. The albino rat is very responsive to the influence of external conditions. The weight increases are greater with abundant and varied diets and smaller when diets are monotonous, although these contain all necessary ingredients. The diet of the animals used in this study was abundant, and consisted of fresh whole milk, lettuce, carrots, bread, cheese and table scraps. Consequently, the average weights in all age groups are very appreciably higher than those given by Donaldson [1924]. Compare, for example, the average weight at 30 days of 71.1 g. for the females, and 77.2 g. for the males in these experiments (average of 130 control animals) with weights of 30.9 and 29.2 g. (Donaldson) and 39.2 and 41.8 g. as reported by Greeman & Duhring [quoted by Donaldson, 1924].

The minimum lethal dose of trimethylamine for the rabbit by subcutaneous injection is 0.8–1.0 g./kg. [Dreyfus, 1920]. Although the intraperitoneal route is far more convenient than the subcutaneous, yet it must be borne in mind that trimethylamine when injected intraperitoneally passes first through the liver. Loeffler [1918] performed extensive experiments with surviving livers of dogs perfused with arterialized blood. He made quantitative determinations of the amounts of urea and  $\text{NH}_3$  in the blood before and after passage through the liver. Adding trimethylamine he found that in passing through the liver it was almost completely transformed into urea. As mentioned before, the doses which can be injected subcutaneously are limited because of the factor of local irritation. Doses of 100–200 mg./kg. of trimethylamine injected intravenously into warm-blooded animals lead only to a transitory fall in blood pressure and slowing of pulse, followed by a slight increase in blood pressure of brief duration [de Waele & de Velde, 1931].

Comparing the average weights of the controls and the test animals at the age of 30 days and taking the weight of the respective controls as 100%, Table 2 shows the comparatively lower percentage weight increases of the experimental animals. The growth retardation is more pronounced with higher dosages, and relatively more with subcutaneous injections. The retardation is slight in absolute figures. The experimental animals are as lively and as vigorous as the controls. It was consistently observed in all experiments that the slight lag in weight increment of the trimethylamine-treated animals, after a number of injections, was always more pronounced in the males than in the females. In other

TABLE 1. Average weight in grams of the control and test animals

		Average weight in g.—at the age of											
		10 days				20 days				30 days			
No. of litters	No. of animals	♀		♂		♀		♂		♀		♂	
		O	T	O	T	O	T	O	T	O	T	O	T
10 mg./kg. subcutaneously	3	21	15.4	15.6	16.1	16.2	20.9	30.6	31.4	31.3	32.4	62.9	66.3
20 mg./kg. subcutaneously	4	23	20.8	21.2	21.3	21.5	41.1	42.0	42.0	40.8	72.8	71.7	73.5
100 mg./kg. subcutaneously	5	22	18.6	18.8	19.3	19.7	37.2	35.0	38.5	39.4	73.1	69.9	80.1
200 mg./kg. subcutaneously	5	21	21.6	23.0	22.4	23.4	40.7	43.5	48.3	45.5	81.1	68.4	85.5
50 mg./kg. intraperitoneally	3	23	17.3	18.4	18.5	17.9	34.1	37.1	38.8	38.1	60.1	65.7	70.3
100 mg./kg. intraperitoneally	3	14	19.6	20.3	20.7	20.8	38.2	38.7	41.6	39.8	68.5	68.9	75.7
Puro controls	2	6	19.3	—	19.8	—	38.6	—	40.6	—	71.2	—	76.8
Total of all animals	25	130	19.2	19.4	19.9	20.2	38.7	38.2	40.8	39.8	71.1	68.3	77.2
Average weight			±3.2	±3.6	±3.3	±3.6	±7.2	±6.1	±7.7	±6.9	±9.3	±7.6	±9.9
Standard deviations													

C=controls. T=trimethylamine-injected animals.

85.8 96.5 95.1  
101.8 106.4  
103.5 121.6 114.4  
98.5  
92.3 97.7  
98.7 90.3  
101.2  
112.4  
101.0 112.3 105.3  
±10.2 ±10.9 ±15.5 ±15.8

TABLE 2

Desigo	Average age in days at start of injections	Average duration in days of injections	% increase of weight at 30 days of age*		The age at opening of vagina days (range in brackets)†		The age at descent of testes days (range in brackets)†	
			♀	♂	C	T	C	T
10 mg./kg. subcutaneously	15	24	100.8	99.4	43 (40-46)	42 (37-47)	26 (24-28)	26 (25-28)
20 mg./kg. subcutaneously	13	20	98.0	94.6	42 (38-45)	42 (37-46)	25 (24-26)	26 (24-27)
100 mg./kg. subcutaneously	15	18	95.6	93.0	40 (37-43)	38 (30-45)	26 (24-28)	20 (24-28)
200 mg./kg. subcutaneously	12	20	84.3	81.7	40 (34-45)	43 (41-49)	26 (21-28)	26 (21-28)
50 mg./kg. intraperitoneally	14	20	109.3	98.4	43 (38-47)	38 (32-41)	27 (25-28)	26 (25-27)
100 mg./kg. intraperitoneally	11	24	100.6	94.3	38 (30-40)	30 (31-49)	26.5 (24-28)	26.5 (24-28)
Pure controls	—	—	—	—	42 (40-43)	—	26 (25-27)	—
Total of all animals	13	21	96.1	91.6	40.7 (34-47)	40.5 (31-49)	26 (21-28)	26 (21-28)

\* The average weight of the respective controls at the age of 30 days is taken as 100%.

† The range covers all individual animals in the various subgroups.

C = controls. T = trimethylamine-injected animals.

words, with equal doses, even though low, trimethylamine is slightly more toxic in the males than in the females, if one considers a slight retardation in weight increment to be a sign of toxicity.

In the immature female rat the external orifice of the vagina is closed by a 'plate' or thin wall of cells. This is ruptured during the first sexual cycle by enlargement of the vagina. In the experiments reported here, this occurred at an average age of 40.7 days in the controls and 40.5 days in the test animals, with a range of 34-47 days in the controls and 31-49 days in the experimental animals. Donaldson [1924] gave as the average age for opening of the vagina 72 days (range 34-109 days), and more recently Rowntree and his collaborators [1935, 1936] report that the vagina opened in their control rats at 55-62 days. The testes descended at the age of 26 days (range 21-28 days) in both control and trimethylamine animals. According to Donaldson [1924] testicular descent occurred at the age of 40 days (no range given) and Rowntree *et al.* [1935, 1936] give the age at 35-40 days. The earlier maturation observed in the experiments reported here, with both treated and untreated animals, is probably connected with the speedier growth and development incident to more favourable conditions, abundant and varied diet, as well as extra good care.

No significant differences in age of testicular descent or opening of the vagina was observed between the controls and the trimethylamine-treated animals. This is a negative result, contrary to the results Havas [1938] reported with cold-blooded animals. The situation is of course not strictly comparable in that his animals (fishes, batrachians) lived completely within a medium containing trimethylamine, whereas the rats received injections only once daily in comparatively low dosages, due to the fact of local irritation. In view of the slight but consistently lower weight increases of the test animals as compared with their litter mates, a histological comparison of their glands of internal secretion, particularly the pituitary glands, may reveal some significant differences.

#### SUMMARY

The effects of trimethylamine (dosages 10-200 mg./kg.) on growth and onset of sexual maturity were studied in twenty-five litters of white rats (254 animals). The trimethylamine-injected animals show no significant difference from the controls in the onset of sexual maturity as indicated by testicular descent and vaginal opening. After a number of injections commencing at approximately 13 days of age, a slight retardation in weight increment was noted. This retardation is more

pronounced in the male animals. At the age of 30 days the females are 96.1% and the males 91.6% of the weights of their litter-mate controls. It is concluded that whatever effect trimethylamine may have upon fishes and batrachians it exerts no hormone-like sex-stimulating effects when injected into the growing white rat.

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THE CHOLINERGIC NATURE OF THE NERVES TO  
THE ELECTRIC ORGAN OF THE *TORPEDO*  
(*TORPEDO MARMORATA*)

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SINCE the first evidence [Dale, Feldberg & Vogt, 1936] for the theory that the transmission of motor-nerve impulses to striated muscles at the motor end-plates is mediated by the release of acetylcholine, many facts have been brought forward supporting this conception. The experiments described in this paper were undertaken in order to discover whether the nerves to the electric organ of the *Torpedo* act in a similar way. This organ, in the *Torpedo* and in some other fishes, may be regarded as a collection of modified motor end-plates lacking the contractile structure of voluntary muscle fibres.

The electric organ of the *Torpedo* consists of a great number of prisms arranged side by side, each prism being built up of about 500 superposed plates, which are covered on their ventral surfaces by a terminal nerve net. At the moment of the discharge the ventral sides of all plates become negative to the dorsal nerve-free sides. Various hypotheses have been formed to explain this special electrogenic phenomenon. Recently,

<sup>1</sup> It was originally intended to publish our experiments in the *Archives Internationales de Physiologie* together with those of Dr Nachmansohn in which he compared the cholinesterase content of the electric organ of different species of fishes and in other tissues of the *Torpedo*. A short communication by all three of us was published in the *Proceedings of this Journal* (1940, 97, 3). In the meantime Dr Nachmansohn has found it more convenient to publish the main part of his results elsewhere (*Science*, 1940, 91, 405; *J. Neuro-Physiol.* 1941, 4, 348). I am therefore publishing the results of the experiments of Prof. Fessard and myself in this *Journal*, publication in the *Archives Internationales* having become impossible. The text is in the main a translation from an agreed French manuscript, with some small alterations and additions which are the outcome of subsequent discussions.

the possibility that the discharge results from release of an electrically active substance has been considered on the basis of researches from two independent sources.<sup>1</sup>

Auger & Fessard [1938, 1939a], working on isolated prisms of the organ, concluded from the latency and form of the discharge following their electrical stimulation that excitation was always indirect. They were able to confirm Garten's [1910] original observation of the impossibility of obtaining a discharge from the whole organ by mechanical or even electrical stimulation after degeneration of its nerves. The fact that the denervated organ was inexcitable even by the stimulus of an electric shock rendered it difficult to imagine that the nerve impulse in the normally innervated organ should transmit its excitation by means of its electrical variation only. From analogy with the action of nervous impulses at motor end-plates of striated muscle they thought the most likely cause for the discharge to be the effect of a polarizing or a depolarizing substance, such as acetylcholine, released by the arrival of the nerve impulse at the terminal nerve net. Results obtained with eserine, curare and atropine on isolated prisms were in agreement with this hypothesis.

The finding of an extremely high concentration of cholinesterase in the electric organ of the *Torpedo* [Marnay, 1937; Nachmansohn & Lederer, 1939] represented the other and more direct evidence in this direction. Marnay & Nachmansohn [1937] had found that the concentration of cholinesterase in striated muscle was greater in those parts of the fibre containing the end-plates, suggesting a high concentration of the enzyme at these regions. As the end-plates are analogous to the elements of which the electric organ of the *Torpedo* is composed, Nachmansohn considered the possibility of an identical mechanism of transmission of the nervous impulses in both structures, and, at his suggestion, Marnay examined the cholinesterase content of the electric organ.

Our experiments were planned to determine whether the electric organ contains acetylcholine; if so, whether the substance is liberated

<sup>1</sup> We are grateful to Sir Henry Dale for drawing our attention to T. R. Elliott's brilliant anticipation of this mode of action. Elliott, who was the first to conceive the idea of chemical transmission in the autonomic nervous system, discussed this possibility for the electric organ in 1914 in his Sydney Ringer Memorial Lecture and even made an attempt to extract the active substance. He writes: 'I have tried in vain to discover an active substance in the muscle plates of striped muscles. And Professor Herring was also disappointed when he examined for this purpose the electrical organs of the skate which are exaggerated motor end-plates... But it is hard to forgo the belief that such discoveries lie in the lap of the future.'

during stimulation of its nerves; and whether an arterial injection of acetylcholine into the organ has an electrogenic effect. In addition, observations were made on the effect of eserine on the discharge.

### METHODS

*Extraction of acetylcholine.* Small pieces of the fresh organ were minced in a mortar with sand in acidified saline solution to which was added eserine 1 in 10,000. The mixture was brought to boiling-point, cooled, made up to a given volume and assayed on the eserinated rectus abdominis muscle of the frog. In some instances the tissue, after being cut into small pieces, was thrown into boiling water containing no eserine, but acidified by HCl to pH of about 4. The mixture was filtered and the residue again minced in a small volume of acidified water with sand, boiled and added after filtration to the main extract, the whole mixture being then made up to a given volume. This extract remained active for several days when kept in the cold.

*Perfusion of the electric organ.* For the study of the effects of arterial injections of acetylcholine and its liberation during nerve stimulation part of the electric organ was perfused. The organ is supplied by four arteries which enter it with the nerves and are accompanied by the veins. Perfusion was carried out from the artery accompanying the second nerve. Injections of Indian ink showed that this artery supplied an area innervated by the first and second nerves, the artery supplying the former being very small. The *Torpedos* were killed by pithing the central nervous system; the electric organ was then cut out, leaving the artery to be cannulated intact and connected with the fish until the last moment. The artery was ligated and cut centrally, thus completely isolating the organ, which was then washed in sea water. A fine glass or metal cannula was tied into the artery and perfusion started at once. In those experiments in which the liberation of acetylcholine was studied the organ was placed in a suitably-shaped paraffin basin which had at its lowest point an outlet for collecting the venous effluent. On the inner surface of the basin a number of small pieces of cork were pinned to keep the organ out of close contact with the basin and prevent retention of fluid. The rate of perfusion was 3-6 c.c./min. The fluid left the organ mainly through the great veins, which were cut in the course of preparation. The fluid was collected in 3-15 min. samples.

The composition of the perfusion fluid varied. In the experiments designed for demonstrating the release of acetylcholine we used first, as recommended by Fühner, a salt solution rich in urea (25 g. urea, 20 g.

NaCl, 1 g. KCl, 1.4 g.  $\text{CaCl}_2$ , 0.83 g.  $\text{MgCl}_2$  and 0.17 g.  $\text{NaHCO}_3$  made up to a final volume of 1100 c.c.). It was found, however, that the urea interfered with the assay of acetylcholine on the leech muscle, and for that reason the fluid was later changed to one containing, in 1100 c.c., 35 g. NaCl, 0.7 g. KCl, 0.1 g.  $\text{CaCl}_2$  and 0.17 g.  $\text{NaHCO}_3$ . In the perfusions made for observing the effects of arterial injections of acetylcholine, the urea was retained but  $\text{MgCl}_2$  was often omitted, and the concentrations of KCl and  $\text{CaCl}_2$  made up to the same values as in the urea-free fluid. Apart from the fact that, in the absence of urea, pronounced oedema developed during prolonged perfusion, there was no difference in the results obtained with the different salt solutions. Eserine, when required, was added to the perfusion fluid in a concentration of 1 in 300,000 to 1 in 150,000.

The venous perfusate was tested for acetylcholine on the eserinated leech muscle. Since the salt concentration of the perfusion fluid was too strong for this purpose, it was diluted with four volumes of distilled water before testing.

*Arterial injections of acetylcholine.* Some extra precautions had to be taken to enable a rapid, close-range arterial injection to be made without mechanical disturbance of the organ. The perfusion cannula, which was also used for the injections, consisted of a syringe needle provided with a tap, the shaft being shortened so that the total dead space was less than 0.05 c.c. and the tip being made blunt and provided with a groove to take the ligature. The needle was fixed and held rigidly in position by a special clamp. The rubber tube leading from the reservoir was attached to the separated nozzle from an all-glass syringe, the tip of which fitted easily into the butt of the needle cannula, and could be quickly removed when necessary. When it was desired to make an arterial injection, the tap of the needle cannula was closed, the glass nozzle from the perfusion system was disconnected; and the nozzle of a syringe, filled with fluid to be injected, was fitted tightly to the butt of the cannula. The tap was then opened, the fluid from the syringe rapidly injected and the tap closed again. A change of nozzles then enabled the perfusion to be promptly restarted, but this was found to be unnecessary between repeated injections of the same solution.

*Stimulation of the nerves.* Since the area perfused from the second artery extended to that supplied from the first nerve, the first two nerves were stimulated together. In large animals the diameter of the nerve trunks was so great as to render it advisable to divide the nerves into finer bundles, thus facilitating a more homogeneous distribution of the

stimulating current. Single or repeated shocks (condensor discharges) were applied to the nerves. For repeated stimulation a rotary commutator was used; the rate of stimulation varied between 2 and 20 per sec., usually about 15. The stimuli were always supramaximal. Use was often made of local mechanical stimulation, which is, in fact, a stimulation of the terminal nerve fibres, in order to test the survival of the perfused organ or to measure roughly the changes in sensitivity during the perfusion. For that purpose light pressure was exerted on the skin with an ebonite rod.

*Observation and recording of discharges.* The electrodes used for leading off were always zinc plates of a few square centimetres, one side being coated with an insulating varnish the other side being placed under the ventral surface of the organ in good contact with the skin. They were connected to the amplifier by flexible leads so as to avoid transmission of the mechanical vibrations set up at the moment of an injection. The changes in potential were led to a direct-current amplifier, but sometimes, to avoid continuous readjustment necessitated by slow changes in polarization and by various other disturbances, we used condenser coupling with a time constant of about 1 sec. The variations recorded following arterial injections of acetylcholine were recorded with this arrangement and have therefore undergone some distortion. The diphasic form of the wave in Fig. 5, for instance, is not a real effect of the injected acetylcholine, but is due to the peculiarities of the recording arrangement.

The amplification was adapted in each case to the size of the deflexion under examination. A sensitivity of  $10\ \mu\text{V}$ . was often necessary for the discharges caused by mechanical stimulation and for the discharges occurring spontaneously under certain conditions. The proper range for detecting the electrical variations caused by small doses of acetylcholine was 0.1–10 mV. Of the total discharge on stimulation of the nerves, a small fraction only was allowed to enter the amplifier. The arrangement allowed quick changes from one sensitivity to another.

Coupled with the amplifier was a Dubois electromagnetic oscillograph for photographic registration and a cathode-ray tube for observing the deflexions during the experiment.

The usual precautions were taken to avoid electrical and mechanical disturbances. The injection experiments were made in a chamber with metal walls. The person making the injections wore rubber gloves, and made certain each time that his movements at the moment of injection did not produce any appreciable variation.

## RESULTS

*Acetylcholine content*

Eserinized saline extracts of the electric organ equivalent to a small amount of fresh tissue had a strong stimulating action when tested on the frog's rectus muscle. No such effect was obtained with saline extracts prepared without eserine or with eserinized saline extracts made alkaline by NaOH and kept in this condition for several minutes. For instance, in Fig. 1 are shown the effects of solutions containing the equivalent of 1 mg. fresh tissue per c.c. of an eserinized saline extract (*B* and *F*), the equivalent of 2.5 mg. tissue per c.c. of the same extract after treatment

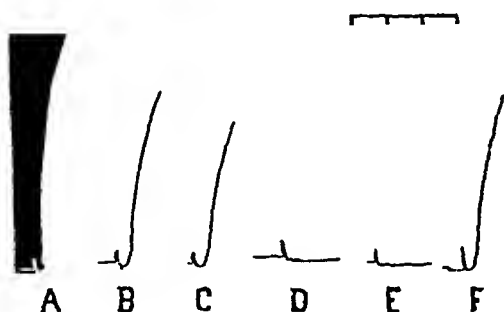


Fig. 1. Responses of the eserinized frog's rectus muscle to acetylcholine chloride (*A* and *C*) and to extracts of the electrical organ. *B*, *D*, *F* = eserinized saline extract; *D* treated with alkali. *E* = saline extract. Time in minutes. (Details see text.)

with NaOH, but neutralized before testing (*D*) and the equivalent of 2.5 mg. tissue per c.c. of a saline extract, made without eserine from another piece of the same organ (*E*). Only the eserinized saline extract, not treated with alkali, caused a contraction which was weaker than that produced by a solution of acetylcholine chloride containing 0.1  $\mu\text{g./c.c.}$  and stronger than one containing 0.067  $\mu\text{g./c.c.}$  (*A* and *C*). In the absence of eserine the active principle was not destroyed when the tissue was extracted with boiling acidified water ( $\text{pH}$  about 4) or with trichloroacetic acid. These facts rendered it highly probable that the active principle was acetylcholine. The conclusion was substantiated by a more complete pharmacological analysis of an extract made with boiling acidified water. The extract was neutralized before testing. Like acetylcholine, small amounts of the extract caused contraction of the eserinized muscle of the body wall of the leech and of the frog's rectus abdominis, inhibited the

stimulating current. Single or repeated shocks (condensor discharges) were applied to the nerves. For repeated stimulation a rotary commutator was used; the rate of stimulation varied between 2 and 20 per sec., usually about 15. The stimuli were always supramaximal. Use was often made of local mechanical stimulation, which is, in fact, a stimulation of the terminal nerve fibres, in order to test the survival of the perfused organ or to measure roughly the changes in sensitivity during the perfusion. For that purpose light pressure was exerted on the skin with an ebonite rod.

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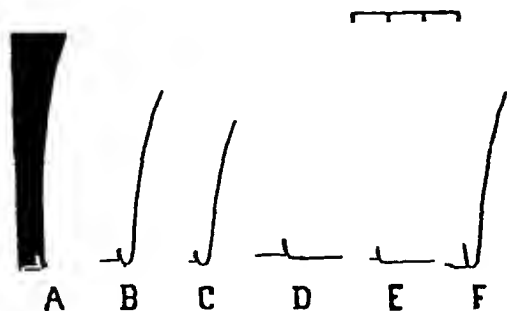


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contraction at *C* was produced by the sample collected during a 3 min. period of stimulation at a frequency of 12 per sec. The 3 min. sample collected immediately after the period of stimulation was again nearly inactive (*D*). The next 3 min. sample was inactive (*E*). This was the usual result. In one experiment only did the sample collected in the first few minutes after the stimulation cause strong contraction, although weaker than that produced by the sample collected during stimulation. When the nerves were restimulated after the venous effluent had again become

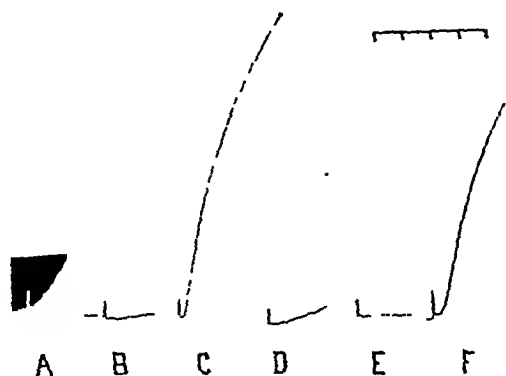


Fig. 2. Responses of the eserinizd leech muscle to acetylcholine 1 in 150 millions (*F*) and to perfusate from electrical organ perfused with eserinizd salt solution. *A* and *B* samples collected before, *C* during, *D* and *E* after stimulation of the nerves. All samples tested in fivefold dilution. Time in minutes.

inactive, the stimulation sample regained its stimulating effect, but this was usually less pronounced than that observed with the sample collected during the first stimulation period.

It appears justifiable to attribute the activity of the stimulation sample to the presence of acetylcholine. The high acetylcholine yield obtained on extraction of the organ is in favour of this conclusion. We have further shown that the active principle released by stimulation was destroyed by alkali and that it did not appear in the absence of eserine from the perfusion fluid. Fig. 3 gives a record of the one experiment done without eserine. Again the first sample had some stimulating effect on the leech muscle (*B*), which diminished greatly with the second sample (*C*). The sample collected during the period of stimulation showed only a slight increase in activity (*D*) which was negligible in comparison with the strong activity of the stimulation sample in the experiment illustrated

frog's heart and lowered the arterial blood pressure of the cat when injected intravenously, and, when injected in larger amounts into the central stump of the coelic artery after evisceration of the cat and giving atropine, caused a rise of arterial pressure due to output of adrenaline from the suprarenals. Atropine abolished the depressor action, the cardio-inhibitor effect, and the contraction of the frog's rectus muscle. There was good quantitative agreement between the depressor, cardio-inhibitor and leech-muscle stimulating actions of the extract, when assayed in terms of the actions of acetylcholine on the same tissues. The extract was inactivated when left for a few minutes with dog's plasma which is rich in cholinesterase; this inactivation did not occur when eserine was added to the plasma. These results appear to justify the conclusion that the active principle was acetylcholine, and for routine estimations it was thought sufficient to assay the eserinated saline extracts on the frog's rectus muscle.

The electric organs were thus found to contain 40-100  $\mu\text{g}$ . of acetylcholine per gram of fresh tissue. In one fish in which the organs weighed 175 g. each, the acetylcholine chloride equivalent was nearly 90  $\mu\text{g}$ ./g., so that the total acetylcholine content of both organs together amounted to about 30 mg. Since the electric organ is an extremely wet tissue, containing 92% of water, the acetylcholine content per unit of dry weight is probably the highest yet observed in any organ.

#### *Liberation of acetylcholine during nerve stimulation*

When the venous effluent from the electric organ perfused with eserinated salt solution was tested in a fivefold dilution on the leech muscle, the samples collected during the beginning of the resting perfusion had some stimulating effect which diminished progressively from sample to sample, until eventually the fluid became inactive. Such an effect was also observed with the first samples of perfusate from the one experiment in which no eserine had been added to the perfusion fluid, suggesting that the effect was possibly not due to acetylcholine. Stimulation of the nerves was not started until the samples had either become inactive, or exerted only very slight effects on the leech muscle. When the nerves were then stimulated for a few minutes, the perfusate collected during the stimulation period caused a strong contraction of the leech muscle, as illustrated by the experiment of Fig. 2. All samples were diluted fivefold before testing. The effects of two consecutive samples collected before the stimulation of the nerves are shown at *A* and *B*. There is a slight contraction at *A*, but none at *B*. The strong

contraction at *C* was produced by the sample collected during a 3 min. period of stimulation at a frequency of 12 per sec. The 3 min. sample collected immediately after the period of stimulation was again nearly inactive (*D*). The next 3 min. sample was inactive (*E*). This was the usual result. In one experiment only did the sample collected in the first few minutes after the stimulation cause strong contraction, although weaker than that produced by the sample collected during stimulation. When the nerves were restimulated after the venous effluent had again become

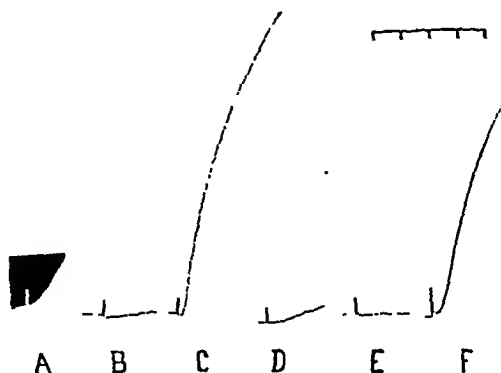


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in Fig. 2. *E* shows the effect of the sample collected immediately after the stimulation.

When the active samples were assayed against acetylcholine it was found that in addition to their direct stimulating action they decreased the sensitivity of the leech muscle to subsequent doses of acetylcholine. The depression lasted for a period of several minutes. The effect has not been further examined, but it rendered an accurate quantitative comparison difficult. It was possible, however, to obtain an approximate estimate of the concentration of acetylcholine in the undiluted samples. In different experiments it varied between 1 in 18 million and 1 in 100 million. In Fig. 2, for instance, the effect of a fivefold diluted sample

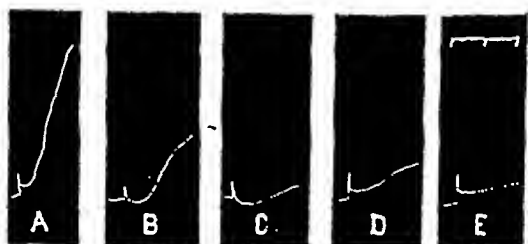


Fig. 3. Responses of eserized leech muscle to acetylcholine 1 in 150 millions (*A*) and to perfusate from electrical organ perfused with non-eserized salt solution. *B* and *C* samples collected before, *D* during and *E* immediately after stimulation of the nerves. All samples tested in fivefold dilution. Time in minutes.

tested at *C* was stronger than that caused by a solution of acetylcholine 1 in 150 million tested at *F*. A closer approximation gave an acetylcholine concentration of the undiluted sample of over 1 in 20 million. Since the venous outflow exceeded 3 c.c./min., the amount of acetylcholine released per minute was at least 0.15  $\mu$ g. Only in the superior cervical ganglion of the cat, an organ in which also the concentration of synaptic junction is very high in relation to the tissue volume, have concentrations of acetylcholine been recovered in the perfusion effluent, during stimulation, of so high an order as those which we observed in some of the few experiments which we were able to make on the electric organ of the *Torpedo*.

#### *Effects of eserine on the nervous discharge from the perfused organ*

The effects of eserine were similar to those obtained by Auger & Fessard [1939a] on the isolated prisms of the electric organ.

After the addition of eserine to the perfusion fluid, the discharges obtained from stimulation of the nerve trunks, or from light pressure on

the skin with an ebonite rod, were modified in a characteristic manner. There was a great prolongation in the descending phase of the electrical deflexion of the single discharge. On the other hand, with continuous stimulation of the nerves to the perfused part, at a frequency of 5-15 per sec., fatigue set in much more rapidly than in corresponding experiments without eserine. In the absence of eserine such repeated stimulations could be continued for 10-15 min. without great diminution in the electrical responses; in the presence of eserine they diminished within 2-3 min. and disappeared after a further 2-3 min. When stimulation was then discontinued for 20-30 sec., the first stimuli on renewal of stimulation produced good responses, but stimulation quickly became ineffective again, and with renewed periods of rest and restimulation, the successive periods of recovered excitability became shorter.

In some experiments the perfusion was started without eserine and, while the arrangement for observing the discharges remained in place, the perfusion fluid was changed to one containing eserine. In these instances we obtained the definite impression that at the commencement of the eserine perfusion, the sensitivity of the organ to stimulation by slight pressure on the skin with an ebonite rod increased; but no accurate quantitative examination of this phenomenon was made. During this period the removal of the pressure from the skin was often followed by a period in which spontaneous discharges occurred, either isolated or in groups. At later stages of a prolonged perfusion with eserine the organ became definitely much less sensitive to mechanical stimulation.

#### *Arterial injections of acetylcholine into the perfused organ*

*Perfusion without eserine.* The arterial injections of relatively strong doses of acetylcholine into the perfused organ, connected with an amplifier, caused a potential change in the same direction as that produced by the normal discharge, but lasting much longer. The sensitivity to acetylcholine varied in different experiments; in some experiments it was necessary to inject 100  $\mu\text{g.}$ , in others effects were obtained with 20 and even with 10  $\mu\text{g.}$  Repeated injections of the same dose gave comparable responses. Control injections of saline produced no changes in potential or slight deviations in the opposite direction. Fig. 4 illustrates the effects of 200, 100, 20 and 5  $\mu\text{g.}$  of acetylcholine (at I, II, IV and V). The first two doses of acetylcholine were injected in a volume of 0.5, the third in a volume of 0.2 and the fourth in a volume of 0.1 c.c. It will be seen that the degree and duration of the electrical variation are dependent upon the dose. In comparing the responses the fact has to be taken into

account that the sensitivity of the amplifier was increased four times after the second injection. The maximal variation after 200  $\mu\text{g.}$  amounted to about 0.7 mV. and after 20  $\mu\text{g.}$  to less than 0.1 mV. 5  $\mu\text{g.}$  were without effect (V).

It is difficult to compare these variations in potential with those obtained during the normal discharge, since we do not know the number of plates involved in the response. The discharge obtained from a small number of plates, by light pressure on the skin with an ebonite rod, produces variations of a few hundredths of a millivolt and requires a great increase in amplification.

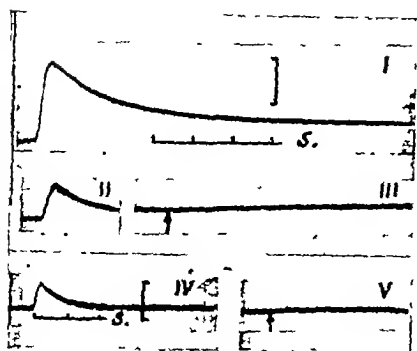


Fig. 4.

Fig. 4. Electrogenic effects of 200, 100, 20 and 5  $\mu\text{g.}$  of acetylcholine (I, II, IV and V) injected arterially into electrical organ perfused with non-eserinized salt solution. At III injection of 0.5 c.c. perfusion fluid. Between II and III sensitivity increased four-fold. 0.5 mV. indicated at I, 0.1 mV. at IV. Time in seconds.

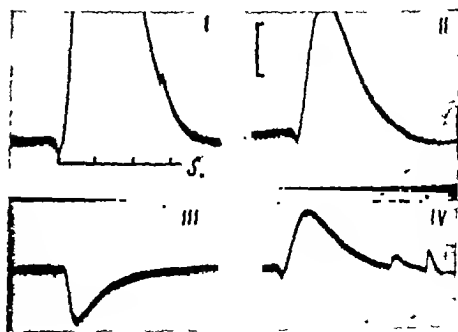


Fig. 5.

Fig. 5. Electrogenic effects of 10, 5 and 2.5  $\mu\text{g.}$  of acetylcholine (I, II and IV) injected arterially into electrical organ perfused with eseriniz salt solution. At III injection of 0.5 c.c. perfusion fluid. 0.5 mV. indicated at II. Time in seconds.

The striking difference is the shortness of the normal discharge, as compared with the long-lasting deviation following an injection of acetylcholine. The normal discharge takes 2-6 msec., whereas the deviation caused by 20  $\mu\text{g.}$  of acetylcholine lasts more than a second, and that caused by 200  $\mu\text{g.}$  has not come to an end after several seconds. With doses over 50  $\mu\text{g.}$  there is always some residual depolarization. With a decrease in dosage the ascending phase of the discharge becomes more abrupt and there is a great shortening of the descending phase.

*Perfusion with eserine.* The effects of acetylcholine were examined during the first stages of perfusion with eserine. Under these conditions the sensitivity of the organ to acetylcholine was found to be much greater

than during perfusion without eserine. The experiment of Fig. 5 shows that under eserine it is possible to obtain an effect even with  $2.5 \mu\text{g.}$  of acetylcholine, injected in  $0.2 \text{ c.c.}$  (IV), the maximal change in potential being  $0.5 \text{ mV.}$  The main wave is sometimes followed by small secondary undulations, such as those seen in the record. In the experiment of Fig. 5 a control injection of perfusion fluid caused a deviation in the opposite direction (III), probably due to the mechanical disturbance, which it is difficult to avoid completely with a rapid injection. The effect becomes smaller when a control injection is made more slowly. It is seen also when acetylcholine is injected, causing a slight deflexion downwards which precedes the response to acetylcholine. It therefore involves a slight deformation of the response, but it has, on the other hand, the advantage of marking the moment of injection and demonstrating the existence of a latency of a few tenths of a second between injection and the start of the acetylcholine response. The change produced by  $5 \mu\text{g.}$  of acetylcholine (II) was stronger than that produced without eserine by  $200 \mu\text{g.}$ , as shown in the experiment of Fig. 4. With  $10 \mu\text{g.}$  (I) the potential change is greater than  $3 \text{ mV.}$ , in fact the height and duration of the deviations in Fig. 5 are shortened a little, since for this experiment the amplifier was coupled with a condenser with a time constant of about  $1 \text{ sec.}$

In two experiments we have used a very sensitive amplification in order to be able to see if there are brief and small discharges superimposed on the slow variation of polarization, which was kept down by inserting a small capacity condenser. Sometimes we observed groups of brief discharges at the moment of the injection of acetylcholine, but the effect was not regularly obtained and occurred sometimes with control injections. It is therefore probably to be attributed to a mechanical effect of the injection. It appears likely that the shock on the adjoining tissue when the arteries are suddenly filled at the moment of injection is sufficient to provoke some rhythmic discharges from a few plates.

## DISCUSSION

Our experiments have confirmed our initial hypothesis of the cholinergic nature of the nerves to the electric organ of the *Torpedo*. The strong concentration of cholinesterase, as shown earlier by Marnay and by Nachmansohn & Lederer, and of acetylcholine in these organs which have a 92% water content, the appearance of acetylcholine in the venous effluent of the perfused organ during stimulation of its nerves



and the electrogenic effect of arterial injections of small doses of acetylcholine, each a suggestive fact in itself, would not separately be sufficient to establish the hypothesis. Taken in conjunction, however, they provide strong evidence in its support and make it possible to accept the idea that the electrical phenomena in these organs are mediated by a cholinergic nervous mechanism.

From these results, viewed in conjunction with the effects of nerve degeneration, the actions of drugs and the latency of the response, we may picture the natural discharge as an effect, on the polarization of the functional interfaces, of minute amounts of acetylcholine released simultaneously at the ventral surfaces of all the plates and then destroyed within the brief refractory period. There is no necessity for postulating a special, independently excitable structure, comparable to the contractile structure of the striated muscle fibre. The liberation of acetylcholine appears to be the final event, devoid of any further stimulating action, and responsible in itself for the sudden change in polarization which constitutes the discharge of the organ.

The effects of eserine on the response to nervous stimulation may be explained on this assumption. If we compare its effects with those obtained on the response of striated muscle to motor nerve impulses [Brown, Dale & Feldberg, 1936; Briscoe, 1936; Bacq & Brown, 1937], there is a striking parallel and a characteristic difference. In both tissues the responses to stimuli repeated at sufficiently short intervals are depressed, those to adequately spaced single stimuli are enhanced, but the enhancement is of a different kind. In the muscle the single twitch is changed into a brief, waning tetanus which can be explained on the assumption that the released acetylcholine, and the consequent change in polarization of the end-plates, persists through several successive refractory periods of the muscle fibre. Only the end-plate being present in the electric organ, no discontinuous discharge can be expected, delay in the destruction of acetylcholine on the active interfaces merely lengthening the wave of potential, particularly in its descending phase. This, in fact, was the observed change brought about by eserine. It is interesting to note that the form of the natural discharge from the electric organ of the ray [Auger & Fessard, 1939b] resembles that from the electric organ of the *Torpedo* after eserine. The cholinesterase concentration in the electric organ of the ray being only a small fraction of that found in that of the *Torpedo* [Nachmansohn, 1940], the resemblance can be explained as due to delayed destruction of acetylcholine in both instances. The rapid onset of fatigue to repeated stimulation after eserine resembles so

closely the paralyzing or curare-like action which eserine exerts on the responses to rapidly repeated nerve impulses in striated muscles, as well as in the sympathetic ganglion [Brown & Feldberg, 1936] as to suggest identical mechanisms in all three instances. For the muscle, as well as for the ganglion, there is good evidence for attributing at least part of the effect to the paralysis produced by excess of acetylcholine, when allowed to act for a sufficiently long time on the motor end-plates or the ganglion cells. We have not as yet made the experiment required to obtain direct evidence for a similar action of acetylcholine on the plates of the electric organ, but the results with eserine are at least suggestive.

If we picture the normal discharge from the electric organ as an action of the released acetylcholine on the state of polarization of the functional interfaces, there is no necessity to expect that an arterial injection of acetylcholine will produce a discontinuous response, as in striated muscle. In striated muscle an injection of acetylcholine causes a repetitive activity in the form of an asynchronous tetanus [Brown, 1937]. The analogy of the continuous change in the state of polarization occurring at the plates of the electric organ, when acetylcholine is injected into its arterial system, strongly suggests that the repetitive series of excitatory waves and corresponding tetanus observed in the voluntary muscle, when acetylcholine is similarly applied, represent the discontinuous response of the fibres to a continuous change in the state of polarization of the end-plates. The same analogy would apply to the contrasted responses of the electric organ and of the voluntary muscle to a single nerve volley under eserine. In the electric organ repetitive discharges are obtained only by repetitive stimulation of the nerve.

The striking difference between the response of the electric organ to a single nerve volley and that produced by arterial injection of acetylcholine is the long duration of the latter, which lasts for several seconds, compared with the few milliseconds of the former. On the other hand, with injected acetylcholine the voltage is much smaller than that of the response of the whole organ to a nerve volley, although it may be several hundred times greater than the partial discharge from a small number of plates, as brought into activity, for example, by exerting light pressure on the organ. To interpret these differences we have to take into account the fact that an arterial injection of acetylcholine is unable to imitate the liberation of acetylcholine occurring at the moment of the nearly simultaneous arrival of an impulse at all the nerve endings. If this liberation represents, as we assume, the final effect of the nerve impulse, the form and duration of the potential deviation will depend

on the rapidity and simultaneity with which the acetylcholine appears and disappears at the active interfaces. Both processes proceed more slowly under the artificial condition of injection than under the normal condition of nervous excitation. The most abrupt injection is long compared with the shortness of a nerve volley. In addition, the acetylcholine, forced through the vessels, will reach the spaces between the plates before reaching the active regions, and, once accumulated in these spaces, or even having impregnated the whole organ, it will take some time to disappear. This process will be further delayed in the presence of eserine. In addition to these factors which are operating on each plate, further lengthening of the response must be caused by temporal dispersion, which exists indeed to some extent even with nervous stimulation, but is naturally much more pronounced with injection, owing to the transmission of the fluid through vessels of different lengths. This can easily be shown by making an arterial injection of Indian ink into the perfused organ, after removal of the skin; the time necessary for the ink to spread through the exposed part is of the order of a second. Such a lack of synchronism would by itself explain the long duration of the deflexion and its relatively small amplitude. The reduction of amplitude in comparison to that of the natural discharge does not result solely from the fact that there is a smaller number of plates discharging at each given moment, and having additive effects, but also, and to a greater extent, from the fact that the mass of the plates inactive at any one moment forms a passive conducting medium, into which the discharges of the active plates may be diverted. Hence only a fraction of the real potential is led off by the electrodes.

It seems useless at the present moment to push further the interpretation of the form of the electrical deflexion produced by injected acetylcholine, since we do not know the number of plates which become activated and we have no data enabling us to state precisely how much of the lengthening of the deviation is due to the factors operating at single plates, and how much to those due to temporal dispersion. The latter certainly play an important role as revealed by the fact that both phases of the deflexion are progressively shortened with diminution of the dose of acetylcholine, with which the number of activated plates decreases. An increase in the duration of the electrical variation on each plate, however, must also play an important role. Apart from the arguments already put forward in favour of this assumption, there is the fact that the slow wave of potential change usually does not show any oscillations. This complete smoothness of the wave is not easily reconciled

with the idea that they are built up by overlapping of single normal discharges of the plates, each having a duration only one thousandth of that of the wave produced by injecting acetylcholine.

New experiments are necessary to interpret quantitatively the character of the response to injected acetylcholine, but there is no inherent difficulty in understanding why the electrical response to it is not repetitive, and why it differs in duration and amplitude from the normal discharge.

The comparison of the electric organ with motor end-plates of striated muscles was to a great extent responsible for the trend our experiments have taken. The evidence, however, once obtained, also throws light on, and gives additional support to, the theory of chemical transmission of motor impulses at the end-plates of striated muscles.

#### SUMMARY

The cholinergic nature of the nerves to the electric organ of the *Torpedo*, indicated by earlier observations, appears to be well established by the following observations:

(1) The electric organ yields on extraction 40–100  $\mu$ g. of acetylcholine per gram of fresh tissue, corresponding to more than 0.1 % of the dry weight.

(2) During stimulation of the nerves to the organ, perfused with an eserinizd saline solution, an alkali unstable substance which stimulates the eserinizd leech muscle appears in the venous effluent. This does not appear in the absence of eserine from the perfusion fluid. It is concluded that the substance so liberated is acetylcholine.

(3) Eserine lengthens the descending phase of the single nervous discharge from the perfused organ and causes rapid fatigue of the response to repetitive nerve stimulation. Similar effects have been obtained by Auger and Fessard on isolated prisms.

(4) An arterial injection of acetylcholine into the perfused organ has an electrogenic effect. When the perfusion fluid contains eserine such an effect can be obtained with as little as 2.5  $\mu$ g. of acetylcholine.

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## THE DETERMINATION OF PLASMA VOLUME BY THE EVANS BLUE METHOD

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THE dye method for the determination of plasma and blood volumes was introduced by Keith, Rowntree & Geraghty [1915]. A measured quantity of dye is injected into the blood stream, and, after an interval for complete mixing, a blood sample is taken for analysis. The plasma volume can then be calculated from the dye concentration in this sample. The dyestuff used must form colloidal aggregates which are unable to pass through the capillary walls. Certain members of the tetrazo class of dyestuffs are very suitable for the purpose, and the earlier workers in the field used red dyes of this class such as vital red, new vital red and congo red. An important advance was the introduction of the blue tetrazo dye T. 1824 or Evans blue by Dawson, Evans & Whipple [1920]. This dyestuff is eliminated very slowly from the blood stream, is non-toxic in concentrations much higher than those normally used, and the blue colour is more readily measured in plasma, especially when the sample to be analysed is slightly haemolysed. Practically all recent studies of blood and plasma volumes with the dye method have used this dyestuff. Those methods in which the dye is determined directly by colorimetric determinations on the plasma itself, have, however, certain marked disadvantages. The turbidity and opalescence of the plasma especially in lipaemic cases, can interfere very seriously with accurate measurement. Harington, Pochin & Squire [1940] have worked out a method in which these difficulties are obviated. In their method, the plasma proteins are digested with pepsin, and the digested sample extracted with *n*-butyl alcohol at pH 3.5 to remove plasma pigments. The sample is then adjusted to pH 1.0, and the Evans blue quantitatively extracted with *n*-butyl alcohol, the colorimetric determination being carried out on the

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butyl alcoholic extract. The method undoubtedly represents a great advance over the direct method, but if a large number of analyses have to be carried out it is slow and tedious. Robinow & Hamilton [1940] have developed a micro method using the alcohol-soluble tetrazo dye brilliant vital red. In this method the plasma proteins and lipides are precipitated with ethyl alcohol leaving a constant fraction of the dyestuff in the clear supernatant fluid. The method, however, requires the injection of relatively large amounts of dye, a procedure which may be inadvisable in certain pathological cases. In addition, the optical properties of brilliant vital red are less favourable than those of Evans blue [Gregerson & Gibson, 1937]. Robinow & Hamilton [1940] state that their method is inapplicable to Evans blue, since the latter dye is 75% precipitated by alcohol under the conditions used by them. Experiments in this laboratory indicate that in very low concentrations Evans blue is almost quantitatively adsorbed by the protein precipitate formed on alcohol precipitation of plasma. The demonstration by Harington *et al.* [1940] that Evans blue is soluble in alcoholic solvents at pH 1, suggested to us that if the plasma proteins were precipitated with acid alcohol, the Evans blue might remain in the supernatant fluid. Preliminary experiments showed that this was so, but the enhanced solubility of the plasma proteins at low pH values resulted in the supernatant fluid being slightly turbid. This difficulty was overcome by the use of an alcohol-soluble protein precipitant. A suitable reagent was finally found in a mixture of hydrochloric acid and alcoholic phosphotungstic acid. This reagent precipitates the plasma proteins, plasma pigments and alcohol insoluble lipides, while the Evans blue remains in the clear supernatant fluid. A simple and rapid method for the determination of plasma volume based on this observation is described in the experimental section.

## EXPERIMENTAL

### *The determination of plasma volume*

#### *A. Injection and sampling technique*

A median antecubital vein is punctured, and 6 ml. of blood withdrawn for control and standard. 5 ml. of a 0.70% Evans blue solution (sterilized by autoclaving) are injected through the same needle, using a calibrated syringe, and the time of completion noted. At intervals of 20, 40 and 60 min., 3 ml. samples of blood are withdrawn from the opposite antecubital vein. All samples are transferred to 15 ml. centrifuge tubes, which have been previously coated internally with paraffin wax, and which

contain approximately 30 mg. of a 2:3 mixture of potassium and ammonium oxalates. The contents of the tubes are mixed by inversion, and centrifuged for 10 min. at 2500 r.p.m. The plasma is immediately separated, and preserved in stoppered tubes for analysis. All syringes used for blood samples must be thoroughly dried and lubricated with medicinal paraffin. The needle should be removed before transference of the blood to the waxed centrifuge tubes, and the blood allowed to flow out under slight pressure only. If these precautions are rigidly adhered to, haemolysis will be obviated.

### B. *Analytical technique*

*Reagents.* (1) Concentrated hydrochloric acid (D. 1.18).

(2) Alcoholic phosphotungstic acid. 15 g. 'Analar' phosphotungstic acid are treated with 100 ml. of 98% ethyl alcohol, the mixture shaken thoroughly and centrifuged to separate the small amount of alcohol-insoluble matter. The amount of insoluble matter appears to vary in different specimens of phosphotungstic acid, but the resulting small variations in reagent concentration have no effect on the precipitation. This reagent is stable for at least 14 days when stored in a dark well-stoppered bottle.

### *Method*

The precipitating reagent is made up by mixing 1 vol. of the concentrated HCl with 6 vol. of the alcoholic phosphotungstic acid and filtering from any insoluble matter. The resulting solution should be absolutely clear. The precipitating reagent is sensitive to light, and if kept for more than 48 hr. slowly develops a blue colour which renders it useless. It is therefore best made up immediately before use.

1 ml. of plasma is measured into a 15 ml. centrifuge tube, and 7 ml. of the precipitating reagent added slowly with vigorous mechanical stirring. Stirring is continued for at least 5 min., and the tube centrifuged for 3 min. at 3000 rev./min. The clear supernatant fluid (approximately 7 ml.) is separated, and the colour measured as described below. Where mechanical agitation is not readily available, satisfactory results may be obtained by vigorous hand shaking for at least 5 min. In this laboratory the five samples necessary for a plasma volume determination are stirred simultaneously. Screw-type all-glass stirrers will be found very suitable for the purpose.

The coloured extracts may be kept in the dark for 24 hr. before measurement, the tubes being tightly stoppered to prevent evaporation of the solvent.



*Control and standard*

1 ml. of undyed plasma is taken through the above process to serve as control for photometric estimations. A 1 : 50 dilution of the injection solution is made up, and 0.100 ml. of this added to 1 ml. of undyed plasma. 1 ml. of the mixture is taken through the routine process to serve as standard. It will be seen that since this plasma standard contains  $\frac{1}{2500}$  of the total amount of dye injected in a volume of 1 ml., it corresponds to a 'plasma volume' of 2500 ml.

*Measurement of colour*

All determinations described in this paper were carried out with the Pulfrich photometer using filter S 61, and 20 mm. cells. If  $P$  is the plasma volume in ml.,  $E_1$  the extinction of the solution measured and  $E_2$  the corresponding extinction of the standard,

$$P = \frac{E_2 \times 2500}{E_1}.$$

The mean value of  $E_1$  for the 20, 40 and 60 min. samples is used in this calculation. Some determinations were also checked using a photoelectric absorptiometer constructed in this laboratory. In this case an Ilford spectrum orange (no. 607) filter was used. Identical values were found.

Some measurements were made with a Dubosq pattern microcolorimeter. The matching of the dilute solutions requires practice, but where great accuracy is not required, reasonably good results can be obtained. The values were within 7% of those obtained with the Pulfrich photometer.

A set of five analyses for a plasma volume determination can be carried out in 35 min., actual manipulations occupying 15 min. of this time.

*Haemolysis*

The method described is not applicable to plasma samples which are more than slightly haemolysed. The acid extraction process forms acid haematin, which is soluble in the reagent, and which has a significant light absorption in the same spectral region as that of the dyestuff. However, by attention to the precautions detailed earlier, haemolysis can be reduced to a level at which no appreciable interference occurs.

*Recovery of Evans blue from plasma*

0.100 ml. of a 0.0014% solution of Evans blue was added to 1 ml. of plasma, and the mixture taken through the routine process. 0.100 ml. of the same Evans blue solution was added to a mixture of 7 ml. of

precipitating agent and 1 ml. of water. The extinctions were measured against corresponding controls using filter S 61 and 20 mm. cells.

Plasma sample extinction	Water sample extinction	% recovery
0.220	0.218	101

The difference is within the error of optical measurement, and recovery made be assumed to be quantitative.

### *Elimination of Evans blue from the blood stream*

It is obvious that if significant loss of the dye from the blood stream occurs during the period necessary for complete mixing, the results will be erroneous. The specimen of Evans blue used in this work (obtained from Messrs Imperial Chemical Industries, Ltd.) had an average elimination rate of 4.8% per hour at the concentration used in this work. This value was obtained from a study of elimination curves observed for 3 hr., since the decrease in the 20, 40 and 60 min. samples is only just above the error of measurement. It is possible, however, that certain pathological conditions may influence the elimination rate, and in such cases correction for elimination should be applied by extrapolation of the elimination curve to zero time. The value so obtained will correspond to instantaneous distribution of the dye in the blood stream.

Dr J. Vaughan<sup>1</sup> has informed us that certain samples of Evans blue are eliminated relatively rapidly from the blood stream, and in view of the observations of Gregerson & Gibson [1937] on the variation in optical properties in different commercial specimens of Evans blue, the need for careful examination of specimens of this dye used for plasma volume determinations must be emphasized.

## RESULTS

Plasma volumes are given in Table 1 for five male and five female normal subjects. These had rested for at least 12 hr. before the determination, and had not been subjected to extremes in temperature. Values have been expressed in terms of ml./kg., and l./sq. m. The surface area was calculated from the Du Bois nomogram [Peters & Van Slyke, 1931].

The series is too small to permit of the deduction of average normal values. The average values are somewhat higher than those found by Gibson & Evans [1937] for a series of ninety normal individuals, but single values fall within the limits found by them. The sex difference found by Gibson & Evans [1937] is also illustrated by our series. Case 7

<sup>1</sup> Personal communication.

shows an abnormally low plasma volume in l./sq. m., but this may be partly accounted for by the abnormal height/weight ratio in this case.

TABLE 1

Case	Sex	Height cm.	Weight kg.	Age	Plasma volume l.	Plasma volume ml./kg.	Plasma volume l./sq. m.
1	M.	170	59.9	40	3.13	52.3	1.85
2	M.	174	67.1	24	3.22	47.9	1.80
3	M.	167	54.9	18	2.80	50.1	1.75
4	M.	166	61.7	28	3.20	51.8	1.90
5	M.	169	68.5	24	3.20	46.7	1.81
					Average 49.8		1.82
6	F.	157	62.0	41	2.72	43.8	1.69
7	F.	157	39.5	19	1.64	41.5	1.24
8	F.	152	65.5	58	2.86	43.7	1.71
9	F.	150	45.4	26	2.50	55.0	1.85
10	F.	160	44.9	17	2.46	54.7	1.72
					Average 47.7		1.64

### *Reproducibility of results*

The reproducibility of values obtained by the method is shown by the following cases in which three determinations were carried out on the same individual at 80 min. intervals.

TABLE 2

TABLE 2					Plasma volume		
Sex	Height cm.	Weight kg.	Age	Time min.	l.	ml./kg.	l./sq. m.
Case 11							
M.	160	52.2	31	0	2.82	54.1	1.86
				80	2.84	54.4	1.87
				160	2.85	54.6	1.88
Case 12							
M.	169	63.7	35	0	3.54	55.6	2.06
				80	3.75	58.9	2.18
				160	3.46	54.3	2.01

### SUMMARY

1. A simple and rapid method for the determination of the dyestuff Evans blue in plasma is described.
2. The method has been applied to the determination of plasma volume.
3. Results are given for twelve normal individuals.

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THE INTERRELATION OF PROSTIGMINE,  
ADRENALINE AND EPHEDRINE  
IN SKELETAL MUSCLE

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WE have recently described experiments made to investigate the transmission of impulses across the synapses of the spinal cord [Bülbring & Burn, 1941]. These experiments were carried out by perfusing the lower half of the spinal cord of a dog with one circulation, and the muscles of the hind-limb by a second circulation. This system enabled substances to be injected into the blood running to the spinal cord without the possibility of them acting on the muscles directly.

In the course of our observations, we discovered that doses of acetylcholine reaching the spinal cord caused a discharge of motor impulses from the cord, and this action was greatly facilitated by the presence of adrenaline. Secondly, when the flexor reflex was elicited by stimulating the posterior tibial nerve and we recorded the contraction of m. tibialis anterior, it was found that the reflex contraction was small, if there was no adrenaline in the blood perfusing the spinal cord; this remained true even when much adrenaline was present in the blood perfusing the hind-limb. However, if adrenaline was added to the blood perfusing the cord, the flexor reflex then became much greater. A third observation concerning adrenaline was also made. When the flexor reflex was recorded, and prostigmine was added to the blood perfusing the cord, the reflex was scarcely affected. This was surprising because when eserine was similarly added, the reflex was greatly increased. We found, however, that when adrenaline was present in the blood perfusing the cord, and prostigmine was injected into it, the injection caused an increase in the flexor reflex similar to the increase caused by eserine.

If these observations are explained as effects of adrenaline on humoral transmission by acetylcholine across the synapse in the cord, then, we argued, a similar effect might be seen in other places where acetylcholine

transmits the impulse. Would it be found that adrenaline affects the action of prostigmine at the neuromuscular junction in the same way as it affects its action in the spinal cord?

### METHODS

Cats were used, anaesthetized first with ether and then with chloralose. The sciatic nerve was divided high up between the thigh muscles and laid upon silver electrodes shielded in a vulcanite fork. Maximal single shocks were applied to it by a neon lamp circuit at rates varying from 4 per min. upwards. The femur was transfixed near its lower end by a steel rod which was held rigidly between clamps. The portion of the os calcis into which the tendon of Achilles is inserted was detached from the rest of the bone, and connected by a stout copper wire to a tension lever. As a rule these preparations were made in the left leg. The right external iliac artery was divided between ligatures, and a cannula inserted into the central end pointing towards the bifurcation of the aorta. The aorta was ligatured below the origin of the external iliac arteries. When injections were made through the cannula, the injected fluid was then carried to the left leg. Injections were also made intravenously.

### RESULTS

*Stimulation at 4 per min.* The contractions of the gastrocnemius recorded in this way are illustrated in Fig. 1, where it is first of all shown (a) that an intravenous injection of 0.04 mg. adrenaline had no appreciable effect on the size of the contractions. In (b) the injection of 0.01 mg. prostigmine by the same route also had no effect, but when it was followed by 0.04 mg. adrenaline, this produced a clear increase. The same effect is shown in (c), (d) and (e) at a later stage, when 0.02 mg. prostigmine itself caused an increase, as it is well known to do. The injection of 0.02 mg. adrenaline, which neither before (c) nor later (e) had an appreciable effect, augmented the contractions when injected at a short interval (5 min.) after the prostigmine.

We observed a similar effect when, instead of prostigmine, we used eserine. For example the injection of 0.05 mg. eserine sulphate caused a small increase of twitch tension, and the injection of 0.01 mg. adrenaline 6 min. later caused a further increase. The effect of adrenaline in augmenting the action of eserine was, however, much less than its effect in augmenting the action of prostigmine. The difference was shown most clearly when adrenaline was given simultaneously. Thus Fig. 2 a shows the effect of 0.05 mg. eserine and 0.02 mg. adrenaline, given together, to

be not much greater than the effect of 0.05 mg. eserine alone, which is seen in Fig. 2 *b*; whereas 0.01 mg. prostigmine with 0.02 mg. adrenaline (Fig. 2 *c*) had a much greater effect than 0.01 mg. prostigmine alone

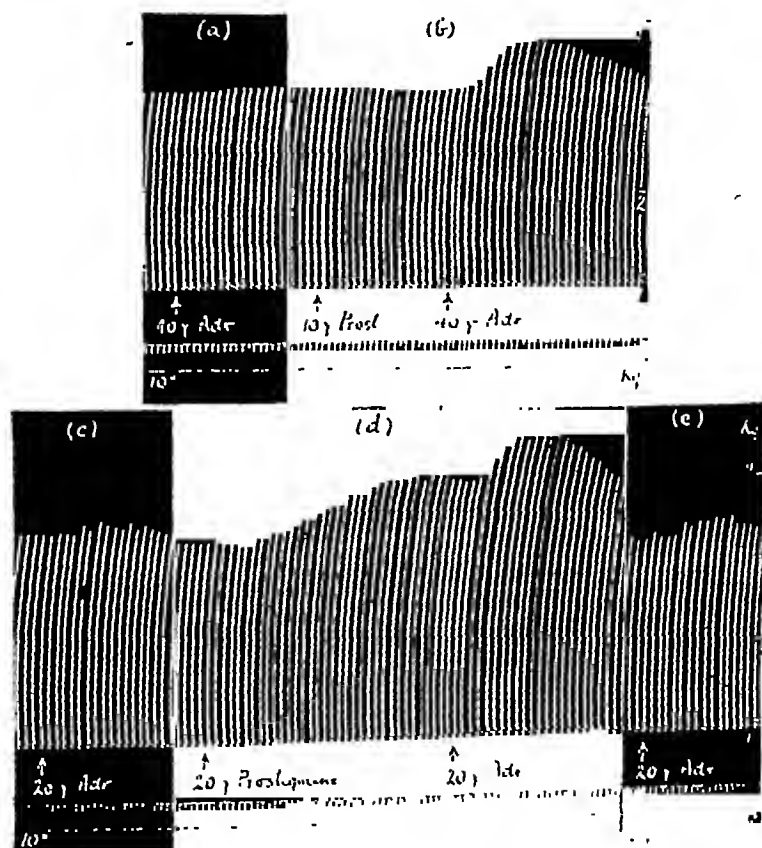


Fig. 1. Cat anaesthetized with chloralose. Contractions of gastrocnemius muscle stimulated by single maximal shocks applied to the sciatic nerve 5 times per min. (a) Shows the effect of an intravenous injection of 40  $\mu$ g. adrenaline before and (b) 4 min. after a dose of 10  $\mu$ g. prostigmine. (c) Shows a slight augmentation of muscular contractions by 20  $\mu$ g. adrenaline. (d) Shows a slow prolonged augmentation caused by 20  $\mu$ g. prostigmine; on top of this augmentation a further increase was produced by injecting 20  $\mu$ g. adrenaline. (e) Effect of adrenaline 15 min. later.

(Fig. 2 *d*). It should be noted that in all experiments in which eserine and prostigmine were compared, a similar increase in tension was caused by doses of prostigmine and eserine in the ratio 1:5, when the doses were given without adrenaline.

The effect of adrenaline on nicotine was also examined. The injection of 1 mg. nicotine tartrate (see Fig. 3 a) slightly depressed the tension evoked by a single shock, and a further slight depression was seen when 0.02 mg. adrenaline was injected  $1\frac{1}{2}$  min. later. There was no striking change in the nicotine effect. If, however, the nicotine and adrenaline were injected together (Fig. 3 b), the depression was much greater.

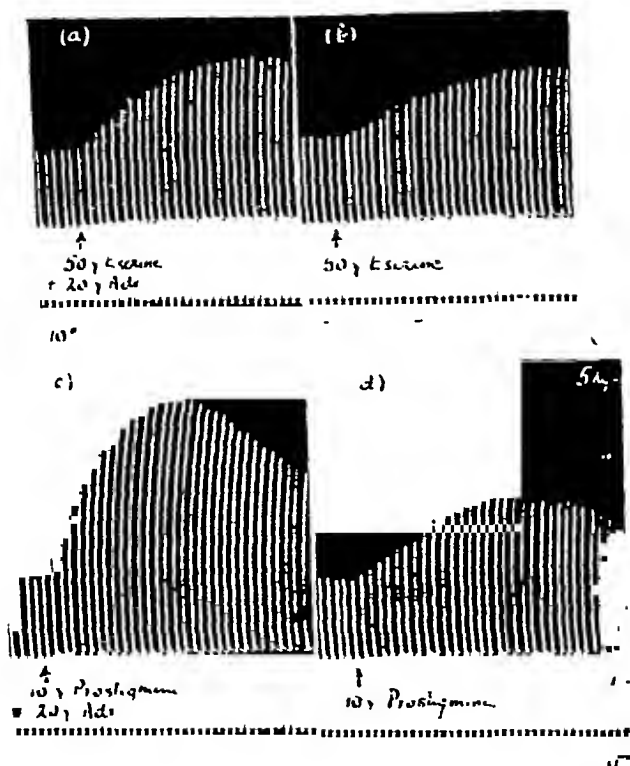


Fig. 2. Records as before. (a) Effect of simultaneous intra-arterial injection of 50  $\mu$ g. eserine and 20  $\mu$ g. adrenaline. (b) Effect of injecting eserine only. (c) Effect of simultaneous intra-arterial injection of 10  $\mu$ g. prostigmine and 20  $\mu$ g. adrenaline. (d) Effect of injecting prostigmine only.

*Single shocks at higher rates.* The foregoing observations were made when single shocks were applied to the sciatic nerve at rates from 4 to 6 times per min. When higher rates, from 15 to 45 per min., were used, a different action of adrenaline was seen, which is shown in Fig. 4, taken from an experiment in which the rate of stimulation was 15 per



min. In (a) the injections of 0.02 mg. adrenaline and of 0.02 mg. prostigmine are seen to have little, if any, effect when given separately. In (b) the simultaneous injection of both substances produced a sharp rise soon interrupted by a depression. In (c) the adrenaline injection was given later than the prostigmine injection which by this time produced on its own account an increase of nearly 50 % in the tension. When, in Fig. 4 c, adrenaline was injected, a clearly defined depression of the prostigmine effect was recorded, so that at this higher rate of stimulation the adrenaline effect was reversed. The rate of stimulation was then reduced to 4 per min., and the injections were made again. Fig. 4 d shows that the augmenting action of prostigmine was still greater, but that the depressant effect of adrenaline was no longer present.

When larger doses of prostigmine, from 0.05 to 0.1 mg., were injected during stimulation at 15 per min., the tension was diminished, as shown in Fig. 5. Adrenaline injected before this diminution had disappeared caused a further diminution, although the same dose injected before the prostigmine caused a small increase, which was probably due to the persistence of prostigmine injected earlier.

*Effects during an interrupted tetanus.* The effect of adrenaline in increasing the tension developed by fatigued muscle has been known since it was described by Gruber [1914]. The evidence [e.g. Bülbiring & Burn, 1940] is mainly in favour of the view that this action is due to

improved neuromuscular transmission, and therefore we made experiments to observe the effect of adrenaline on fatigued muscle in the presence of prostigmine. We perfused the hind-leg of a dog with defibrinated blood, stimulated the sciatic nerve with condenser discharges at the rate of 400 per sec. applied 120 times per min. for a duration of 0.02 sec. The contractions of the gastrocnemius muscle were recorded by

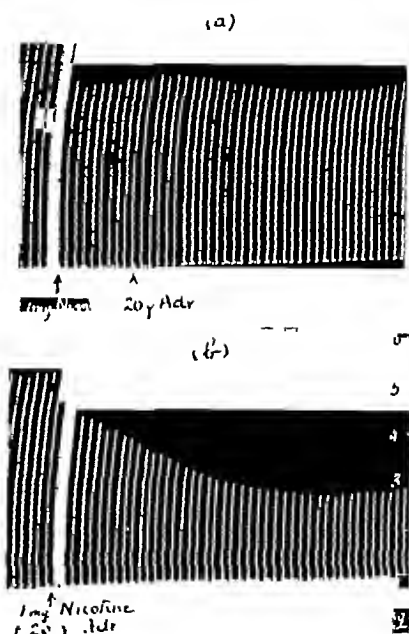


Fig. 3. Records as before. (a) Effect of intra-arterial injection of 1 mg. nicotine followed by 20 µg. adrenaline. (b) Effect of simultaneous injection of these doses of nicotine and adrenaline.

a tension lever. When the stimulation had been applied for several minutes the contractions, which had diminished rapidly at first, remained at a uniform height, and the injection of 0.008 mg. adrenaline into the

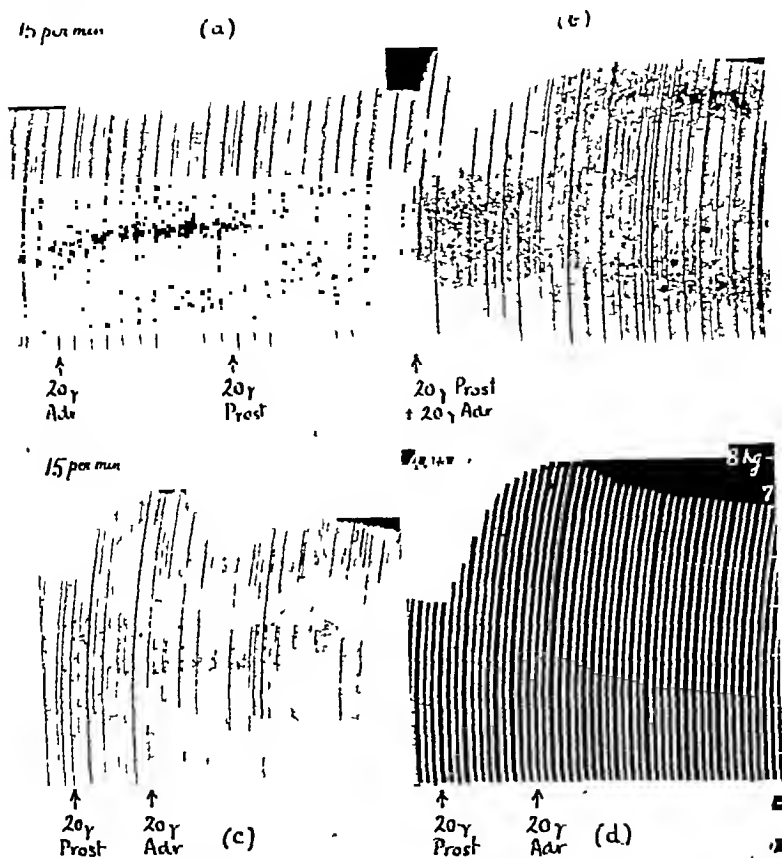


Fig. 4. Records as before. (a) Fifteen maximal shocks per min. Very slight changes in the size of muscle contractions after injections of 20  $\mu$ g. adrenaline and 20  $\mu$ g. prostigmine. (b) Sharp rise interrupted by depression after simultaneous injection of prostigmine and adrenaline. (c) Analysis of the effect observed in (b) by injecting the adrenaline after the prostigmine. (d) Same sequence of injections at slower rate of stimulation when adrenaline caused no depression.

arterial cannula now caused the increase shown in Fig. 6 a. In Fig. 6 b is shown the effect of injecting 0.02 mg. prostigmine, which diminished the contractions. In Fig. 6 c the adrenaline injection was repeated and caused a diminution. The conditions here were, however, different from the conditions when the nerve was stimulated with single shocks at

15-45 per min. With that stimulation, whether prostigmine increased (Fig. 4 c) or decreased (Fig. 5) the contractions, a subsequent dose of

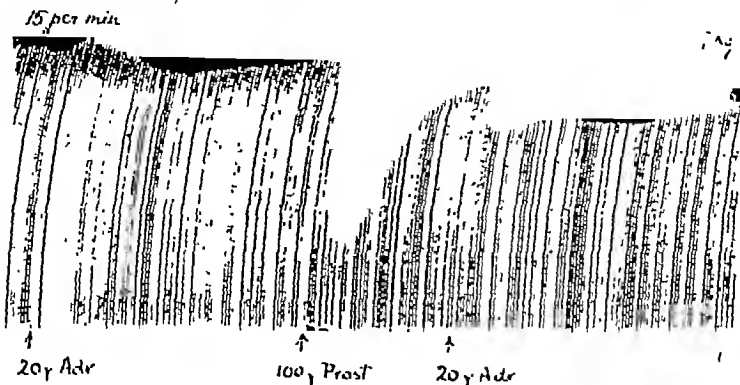


Fig. 5. Records as before. Fifteen maximal shocks per minute. (Two doses of 0.1 mg. prostigmine had been injected 1 hr. before.) The injection of 20  $\mu$ g. adrenaline caused a small increase of muscle contractions, 100  $\mu$ g. prostigmine depressed them, and, after this, 20  $\mu$ g. adrenaline caused a further depression.

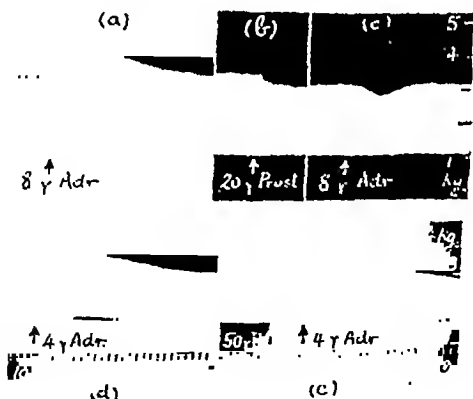


Fig. 6. Dog. Perfused hind-leg. 120 tetani per min.; muscle fatigue. (a) Injection of 8  $\mu$ g. adrenaline into arterial cannula caused, after a small initial diminution, augmentation of muscle contractions of 1 kg. (b) 20  $\mu$ g. prostigmine was given, after which (c) adrenaline caused depression only. (d) (from another experiment) shows the big increase of muscle tension after the injection of 4  $\mu$ g. adrenaline and (e) a much smaller effect after 50  $\mu$ g. prostigmine had been given.

adrenaline only depressed them. With interrupted tetani, adrenaline at an early stage after prostigmine was still able to cause an increase as shown in Fig. 6 e, though a smaller increase than before (Fig. 6 d).

*The action of ephedrine.* Since ephedrine has been used in the treatment of myasthenia gravis, both alone [Edgeworth, 1930] and in conjunction with prostigmine [Viets & Schwab, 1939; Schleizinger, 1940], we were anxious to see if ephedrine augmented the action of prostigmine as did adrenaline. We injected ephedrine during the increased tension

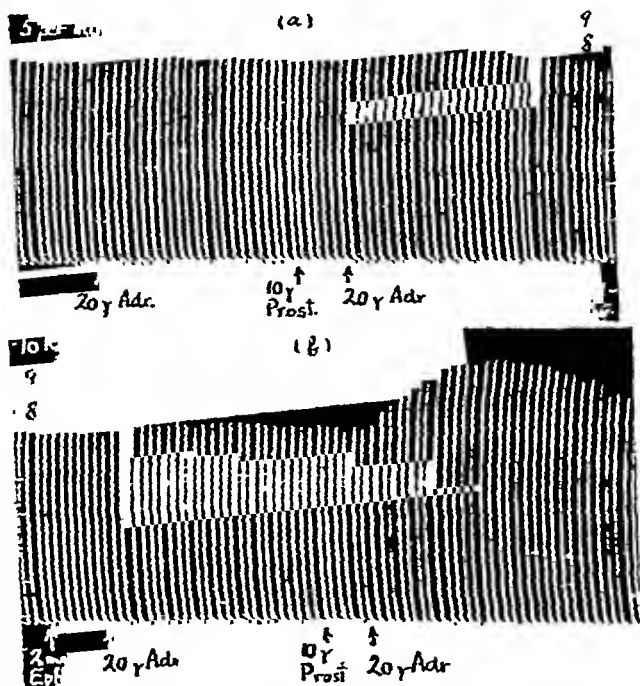


Fig. 7. Cat, chloralose. Response of gastrocnemius to maximal shocks to sciatic nerve 5 per min. (a) Shows very slight changes in the size of muscular contractions due to adrenaline injected before or after prostigmine. (b) Shows that 2 mg. ephedrine did not affect the action of adrenaline or prostigmine itself, but after prostigmine adrenaline now caused an augmentation.

produced by prostigmine when the rate of stimulation was 4 per min., but observed no augmentation, although adrenaline injected 3 min. later caused the usual increase. It seemed that ephedrine had no direct relation to the action of prostigmine. We then found that after the injection of ephedrine, adrenaline was more effective in augmenting the action of prostigmine than before. An example of this is given in Fig. 7. In (a) the injection of 0.02 mg. adrenaline, of 0.01 mg. prostigmine and of 0.02 mg. adrenaline 1 min. later were alike without effect. In (b) 2 mg. ephedrine

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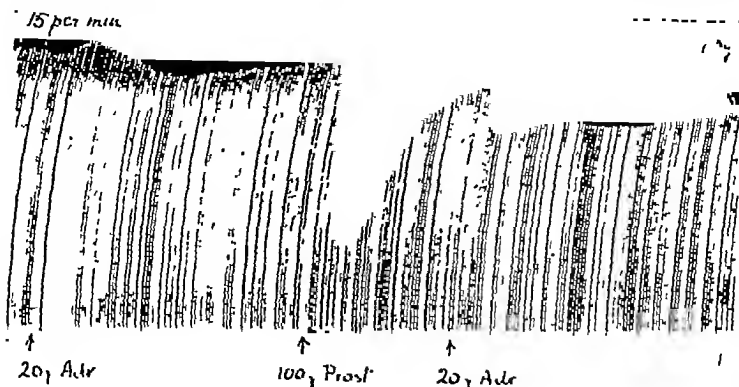


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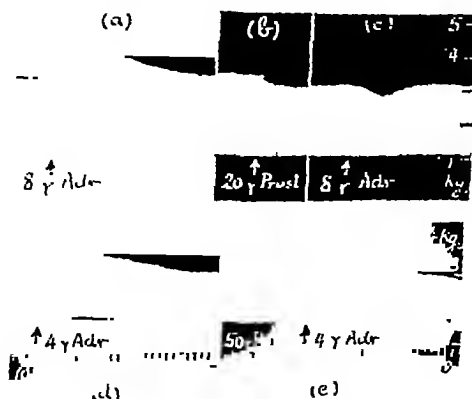


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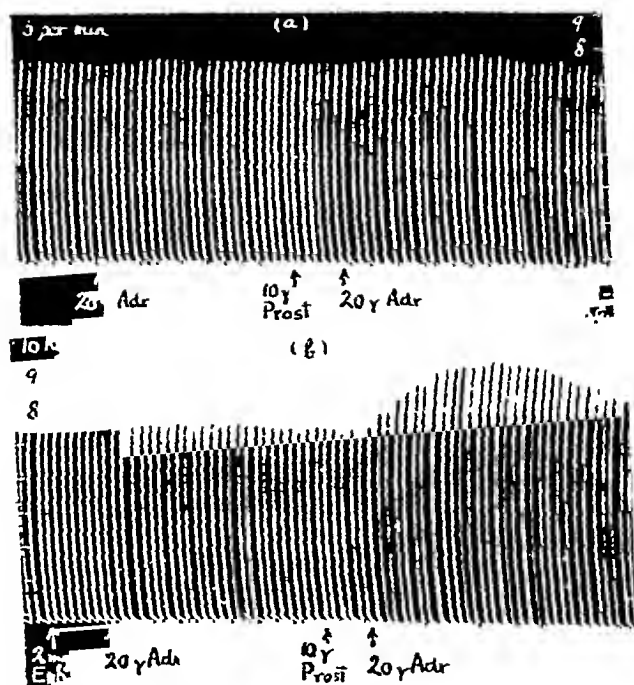


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hydrochloride were first injected. This had no effect itself, or on the subsequent injection of 0.02 mg. adrenaline. It did not affect the response to 0.01 mg. prostigmine. The response to 0.02 mg. adrenaline injected after the prostigmine was, however, greatly increased by the injection of ephedrine.

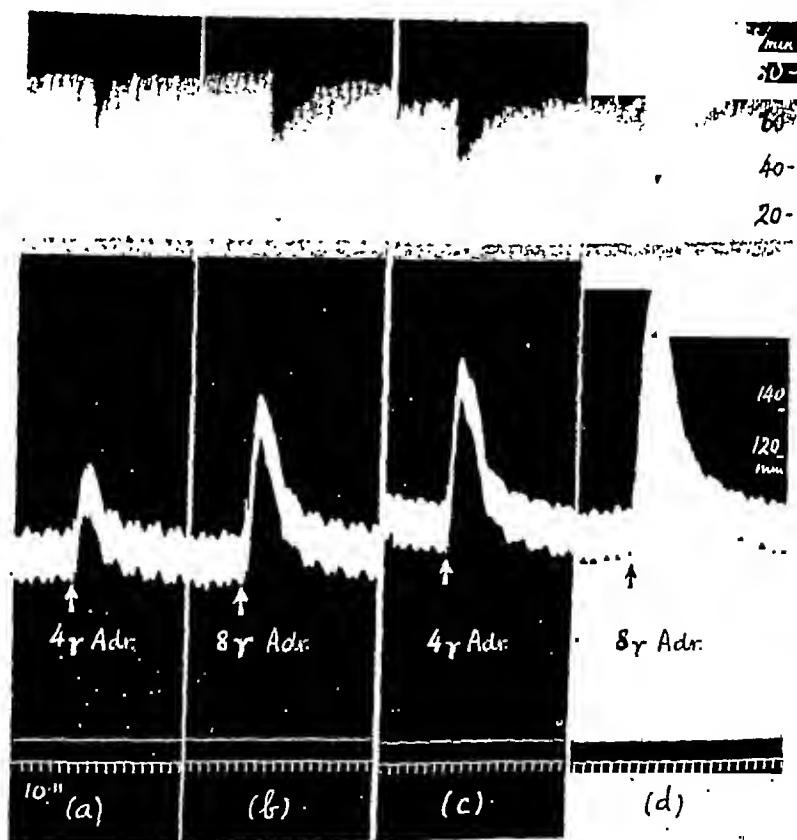


Fig. 8. Dog, hind-leg perfusion. Top: Venous outflow, bottom arterial pressure. (a) and (b) show vascular effect of 4 and 8  $\mu$ g. adrenaline before and (c) and (d) after 20  $\mu$ g. prostigmine.

Of other sympathomimetic substances we tested dihydroxyphenyl-ethylamine and also *l*-meta-sympatol. This substance is identical with adrenaline save for the absence of the OH group in the para position in the ring. The former substance was inactive, while sympatol had the same augmentor action as adrenaline when given in a dose 8–10 times as great.

*Adrenaline and prostigmine on the blood vessels.* In the course of these experiments we have observed an effect of prostigmine on the vasoconstrictor action of adrenaline which is relevant to this paper because it indicates that these two substances affect one another directly. Mendez & Ravin [1941] have investigated the action of prostigmine on the circulation, and have observed that prostigmine causes a rise in blood pressure, well shown after atropine. They conclude that constriction of some portions of the peripheral vascular system must be partly responsible for this effect. We have observed that prostigmine increased the tone of the vessels of the dog's hind-leg perfused with defibrinated blood, and increased the constrictor action of adrenaline. Fig. 8 *a, b* show the constriction caused by 0.004 and 0.008 mg. adrenaline before the injection of 0.02 mg. prostigmine. Shortly after, the arterial tone rose and the effect of these doses of adrenaline was approximately doubled (Fig. 8 *c, d*).

#### DISCUSSION

In the foregoing account we have described that if, in a cat under chloralose, the sciatic nerve is stimulated at a slow rate, such as 4 times per min., with maximal single shocks, the twitch tension is augmented by adrenaline if a dose of prostigmine has been injected a few minutes before. The same phenomenon is seen after a dose of eserine, though the effect is smaller. If higher rates of stimulation, 15–45 per min., are employed, the increased twitch tension produced by the injection of prostigmine is diminished by the injection of adrenaline. It is well known that if the muscle is tetanized to fatigue, the tension is augmented by adrenaline; we now find that if prostigmine is injected first, the adrenaline augmentation is diminished or abolished.

We think these observations are best explained by reference to the observation made by Dale & Gaddum [1930] that when a strip of the denervated diaphragm of a kitten was suspended in a bath, the addition of acetylcholine caused a contraction, but that in the presence of adrenaline, which by itself had no effect, this action of acetylcholine was greatly enhanced. This observation leaves no doubt that adrenaline potentiates the action of acetylcholine in skeletal muscle, and does so by a mechanism which is independent of the circulation. Vascular effects are not concerned.

There are now two conditions known in which adrenaline augments the contraction of skeletal muscle in the body. The first is during fatigue, in response to tetanic stimuli applied to the nerve, as demonstrated by Gruber [1914]. The action of adrenaline is almost certainly the same as the action of the sympathetic nerves described by Orbeli [1923]. The



second is in unfatigued muscle when prostigmine has been injected a short time previously. What is the common feature of these two conditions? It is that in both a larger amount of acetylcholine than usual appears at the motor end plates. In the fatigued muscle this comes from the very frequent stimuli reaching the nerve ending, and liberating the acetylcholine; in muscle into which prostigmine has been injected, stimulated at infrequent intervals, the acetylcholine accumulates because the prostigmine prevents its rapid destruction. We suggest that adrenaline increases the height of muscle contraction in both conditions by potentiating the effect of this increased amount of acetylcholine.

In the fatigued muscle the potentiating effect of adrenaline is diminished, then abolished, and finally converted to a depression by the injection of prostigmine. In the infrequently stimulated muscle treated with prostigmine, the potentiating effect of adrenaline is converted into a depression by increasing the rate of stimulation. That is to say, in both conditions, when too much acetylcholine is produced, the effect of adrenaline is again to intensify its action and to produce the effect of excess, which is depression.

While recognizing that adrenaline does potentiate the action of acetylcholine, and that this fact may explain all the phenomena, we think that the possibility that adrenaline modifies the action of prostigmine should be borne in mind. A direct relation between adrenaline and prostigmine is suggested by several facts. Both prostigmine and eserine increase the tension developed in the muscle in response to single shocks applied to the nerve; yet adrenaline augments the effect of prostigmine very much more than it augments that of eserine. It was, indeed, a difference of this kind in the spinal cord that led to these experiments, for while eserine readily increased the flexor reflex, prostigmine had this effect only in the presence of adrenaline. On the blood vessels, where acetylcholine is certainly not involved, prostigmine causes some vasoconstriction and augments the constrictor action of adrenaline. It seems clear that the interaction of these two substances is not confined to the potentiation by adrenaline of the acetylcholine protected by prostigmine, but that adrenaline somehow increases the action of prostigmine itself.

Our observations on ephedrine show that this substance has no action like that of adrenaline in relation to prostigmine, and does not augment the muscle tension in the presence of prostigmine. The effect of adrenaline on this tension is, however, greater after ephedrine has been injected, presumably because the rate of adrenaline destruction is then slower.

In these observations we have a possible explanation of the finding

of Edgeworth [1930] that ephedrine benefits sufferers from myasthenia gravis. If adrenaline plays a part in normal muscle contraction (and our observations make it more likely that it does), ephedrine will benefit sufferers from myasthenia gravis by prolonging the effect of adrenaline naturally produced in the body, either from the suprarenal medulla or at sympathetic nerve endings.

The use of ephedrine in myasthenia has been obscured by Walker's discovery of the value of prostigmine [1935], but recently Viets & Schwab [1939] and Schlezinger [1940] have stated that it is better to give ephedrine in addition to prostigmine than to give prostigmine alone. Our observations agree very well with this clinical experience.

### SUMMARY

1. If contractions of the gastrocnemius of a cat are elicited 4-6 times per min. by single maximal shocks applied to the nerve, the increase of tension produced by the injection of prostigmine is very much greater if adrenaline is given together with prostigmine.
2. The increase due to adrenaline is also evident if the adrenaline is given some minutes after the prostigmine.
3. Adrenaline augments the effect of eserine much less than it augments the effect of prostigmine.
4. When the rate of stimulation is raised to 15-45 per min., prostigmine increases the tension, but adrenaline then decreases it.
5. Ephedrine has no action like that of adrenaline in relation to prostigmine; but after the injection of ephedrine, adrenaline has a greater effect than before.
6. The relation of these and other observations to the Orbeli phenomenon and to myasthenia gravis is discussed.

Our thanks are due to Roche Products, Ltd., for a supply of prostigmine.

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THE ACTION OF THE OXYTOMIC HORMONE OF THE  
PITUITARY GLAND ON URINE SECRETION

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THE diuretic and antidiuretic actions of posterior-pituitary extract are well known. The action elicited by an injection of the extract varies with the quality of the urine being secreted. In general it has been observed that when the osmotic pressure of the urine is low pituitary extract exerts an antidiuretic action, and when the osmotic pressure is high it exerts a diuretic action.

Early work with whole pituitary extract by Stehle & Bourne [1925] and Stehle [1927] has shown that both diuretic and antidiuretic actions are accompanied by increased urinary excretion of certain salts. This led to the suggestion that the diuretic action was not a direct action on water excretion, but an action on salt output; hence, if pituitary extract is given when the salt content of the urine is already high, the increase in salt excretion necessitates an increase in water output.

This theory that pituitary diuresis is actually a salt diuresis was supported by later workers. Thus Nelson & Woods [1934], working with mice, found that small doses of pituitary extract inhibited water diuresis, but on increasing the dose the augmented salt output was accompanied by an increase in water excretion. Unna & Walterskirchen [1936] found that an increase in chloride concentration of the urine is produced by smaller doses of a pressor preparation than are necessary to increase the volume of urine. Melville [1936], from a study of the effect of salt saturation on the urinary response to pituitary extract, concluded that the diuretic and antidiuretic responses are unrelated and designates them as 'salt-mobilizing' and 'water-retaining' actions.

After methods had been devised for the separation of the oxytomic and pressor principles of pituitary extract [Kamm, Aldrick, Grote, Rowe & Bugbee, 1928; Stehle, 1933] studies were carried out to determine the principles responsible for the various actions of whole extract. Bugbee

& Simond [1928], using the preparation of Kamm *et al.* [1928], concluded that the pressor principle has both diuretic and antidiuretic actions in anaesthetized rabbits. Macdonald [1933] states that in the cat, dog, rabbit and man, pituitary diuresis is only produced by doses which affect the circulation. Melville & Holman [1934], using a more sensitive technique, studied pituitary diuresis in urethanized rabbits. From a comparison of the activities of four preparations, the commercial preparations, pitressin and pitocin, and Stehle's [1933] preparations, postlobin-V and postlobin-O, they concluded that the diuretic effects observed suggest that these are due to the pressor principle. Using unanaesthetized bladder fistula dogs, Stehle [1934] has presented convincing evidence that the pressor and antidiuretic actions of pituitary extract are caused by the same principle. Other workers support this conclusion, the only noteworthy evidence to the contrary being that of Heller [1939, 1940]. Recent experiments [Fraser, 1941], however, lead the writer to believe that Heller's conclusions are ill-founded.

A survey of the literature, which is discussed incompletely above, indicates that the pressor principle has generally been regarded as responsible for the increase in water and salt excretion which, when conditions are suitable, follows the administration of posterior-pituitary extract. Draper [1929] presented experiments from which he concludes that a third substance, different from the oxytocic and pressor hormones, causes the diuresis after pituitary extract. His data, however, appear rather unconvincing. Recently, the writer reported experiments [Fraser, 1937] on rats which showed that preparations of the oxytocic hormone exerted a diuretic action which could not be accounted for by their contamination with pressor hormone. The chloride concentration during this diuresis was unchanged. These observations have been confirmed by Kuschinsky & Bundschuh [1939]. Boyd, Garand & Livesey [1939], measuring the rate of water loss in hydrated animals, find that very large doses of pituitrin increase it, medium doses inhibit it and very small doses again increase the rate of this loss. In view of our results it seems probable that the diuretic action of the smallest doses employed by these workers was due to the oxytocic hormone.

These recent papers on the diuretic action of oxytocic extracts lead to considerable confusion regarding the constituent or constituents of pituitary extract responsible for the increased water and salt excretion. The present work consists of an attempt to clarify this confusion and to make a more complete study of the action of oxytocic extracts on urine secretion.

During the course of this work it was observed that the oxytocic extract was very active in decreasing urinary phosphorus excretion, and these data are included in this paper. Previous workers have studied the effect of posterior-pituitary extract on the phosphorus of the urine and blood. Stehle & Bourne [1925] found that phosphorus was excreted at an increased rate during pituitary diuresis in dogs. Bollinger & Hartman [1925] observed increases in blood phosphates in dogs after the intravenous injection of pituitary extract, while Gollwitzer-Meier [1926] confirmed this result using rabbits. All this work was performed before a method for the separation of the posterior-pituitary hormones had been devised, and therefore whole pituitary extract was employed. The doses used in this early work were quite large, ranging from 10 units upward. Brull & Eichholtz [1926] studied the acute effect of removal of the pituitary body and of injury to the tuber cinereum on the urinary excretion of inorganic phosphorus. In the majority of both types of experiment the phosphorus excretion was abolished, but the administration of pituitrin had inconstant and doubtful effects in restoring the excretion. Anderson & Oastler [1938], in chronic experiments, have observed that hypophysectomy in the rat markedly lowers the inorganic phosphate of the plasma.

### METHODS

*Preparations.* Postlobin-V and postlobin-O, Stehle's [1933] highly purified preparations of the pressor and oxytocic hormones respectively, were used in this work. The actual extracts employed were prepared in this laboratory [Stehle & Fraser, 1935]. Postlobin-V contains 200 pressor units and 10 oxytocic units per mg. and postlobin-O contains 250 oxytocic units and 5 pressor units per mg. Therefore, if one administers one unit of postlobin-V, 0.05 oxytocic unit necessarily accompanies this dose; similarly, one oxytocic unit of postlobin-O would be contaminated with 0.02 pressor unit.

*Chemical methods.* Chlorine determinations were made by digesting the urine in concentrated nitric acid, containing silver nitrate. Filtering and weighing the silver chloride was carried out according to the method of Pregl [1930]. Total phosphorus determinations were made by washing with sulphuric and nitric acids, and hydrogen peroxide, followed by precipitation and weighing according to Pregl [1930]. Hydrogen-ion concentrations were determined using bromcresol purple and phenol red. Clark & Lubs [1916] series of buffers were employed in making the comparisons.

## RESULTS

*Effect of pituitary extracts on rate of water excretion  
in hydrated animals*

The ability of postlobin-O to accelerate water excretion in hydrated rats was noted in our earlier paper [Fraser, 1937]. The experiments described below, however, demonstrate this action more clearly.

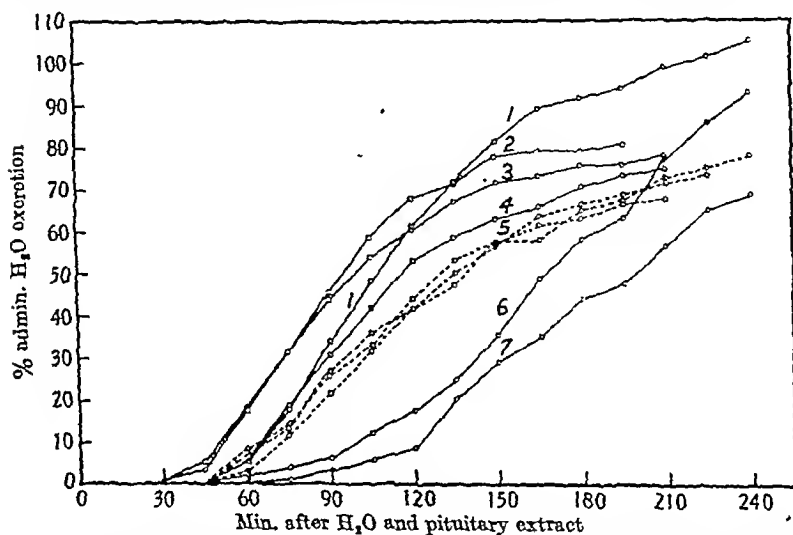


Fig. 1. (Doses in this and later figures expressed in units per 100 g. rat.) Exp. 1: 0.05 oxytomic unit postlobin-O (0.001 pressor unit as contaminant). Exp. 2: 0.005 oxytomic unit postlobin-O. Exp. 3: 0.001 oxytomic unit postlobin-O. Exp. 4: 0.0001 oxytomic unit postlobin-O (0.005 pressor unit as contaminant). Exp. 5 (broken lines): 3 saline controls. Exp. 6: 0.25 oxytomic unit postlobin-V. Exp. 7: 0.001 pressor unit postlobin-V.

The technique employed was similar to that of Burn [1931]. Groups of sixteen adult male rats were used. After fasting the animals overnight, they were given 5% of their body weight of water by stomach tube, and immediately injected subcutaneously with the pituitary preparation dissolved in saline. Readings of urine volume were made every 15 min., and these values were translated into percentages of administered water. The results are expressed graphically in Fig. 1. The controls (broken lines, 5) shown represent those of Exps. 2, 3, and 4 and were carried out according to Burn's [1931] assay technique of switching groups. All these experiments were performed on the same two groups of sixteen rats each, chosen at random from the same colony. Repeated controls remained quite constant. These control excretion rates appear much

slower than those of most other workers because the former are plotted in terms of percentage of administered water and not of percentage of total water excreted. Exp. 7 shows that 0.001 pressor unit of postlobin-V produces a good antidiuretic action, whereas in Exp. 1, 0.001 pressor unit and 0.05 oxytocic unit of postlobin-O produces the opposite action on water output—a marked accelerating action. In Exp. 6, 0.005 pressor unit and 0.25 oxytocic unit of postlobin-O had some antidiuretic action, but this is less than the effect of one-fifth this pressor dose of postlobin-V, shown in Exp. 7. Exps. 2-4 show the accelerating effects of smaller doses of the oxytocic extract. Obviously there is something in postlobin-O, presumably the oxytocic hormone, which accelerates water excretion, and which antagonizes the antidiuretic action of the contaminating pressor hormone. Antidiuretic action, however, appears to be the dominating one if large doses of postlobin-O are employed. An observation not shown completely in Fig. 1 should be mentioned; when the larger doses of postlobin-O were given as in Exps. 1 and 6, the excretion curves flattened out only after passing the 100% value, whereas with smaller doses of postlobin-O, with controls and with postlobin-V the curves flattened out at about the 80% value. In other words, these experiments also showed that something in postlobin-O not only accelerates water output, but also increases the total volume of excretion.

*Effect of pituitary extracts on the excretion of water  
and chlorides in non-hydrated animals:*

*Experiments with rats.* These were carried out on a group of sixteen adult male rats kept on a constant diet of Purina Fox Chow. They were fed once daily at 5.30 p.m. The amount of food was adjusted so that they ate it all quickly, but was sufficient to keep their weight almost constant. On the morning of the day of the experiment they were given 1% of their body weight of water by stomach tube and the drinking bottles were removed from their cages. Four hours later they received subcutaneous injections of pituitary extract in a volume of 0.2 c.c. saline/100 g. body weight. During the subsequent 4 hr. urine was collected by placing the four cages, containing four rats each, over glass funnels. The collections were made under oil in graduated cylinders. At the end of the experiments food and water were again placed in the cages. At least a day was allowed to elapse between experiments. However, controls were done occasionally between experiments and were found to be unaffected by the pituitary injection of the previous day.

The accompanying Figs. 2-5 were constructed using calculations made from the data in Table 1 and from the values for the pressor and oxytocic activities of postlobin-O and postlobin-V found under 'Methods'. The remainder of the graphs were constructed from other data.

TABLE 1

	Oxytocic units P-O per 100 g. rat								Pressor units P-V per 100 g. rat		
	Saline only			0.0001	0.001	0.01	0.1	1.0	0.002	0.02	0.2
	11.1	10.3	10.9	13.9	17.5	32.5	63.5	71.0	9.0	9.5	25.4
C.C. urine from 16 rats in 4 hr.	10.3	10.2	11.9	12.2	15.2	31.1	62.6	—	9.5	8.8	19.4
	12.2	11.6	11.5	12.6	15.4	33.2	68.1	—	11.7	11.2	32.8
Mg. Cl in above urine samples	43.4	36.6	52.4	81.0	77.5	168.0	298.4	447.1	—	42.8	229.1
	35.6	36.5	55.7	51.4	77.1	144.6	261.7	—	63.6	62.9	205.6
	49.0	42.6	43.6	30.5	60.5	128.8	294.2	—	58.7	59.6	351.0
Av. conc. Cl in mg./c.c.	3.80			4.15	4.48	4.57	4.40	6.30	5.35	5.66	10.11
pH values of above urine samples	6.4	6.3	6.2	6.4	6.6	7.0	7.2	7.3	6.3	6.7	6.8
	6.2	6.3	6.2	6.5	6.4	6.9	7.1	—	6.3	6.2	6.8
	6.3	6.3	6.4	6.6	6.6	6.8	—	—	6.3	6.5	6.9

P-O = Postlobin-O.

P-V = Postlobin-V.

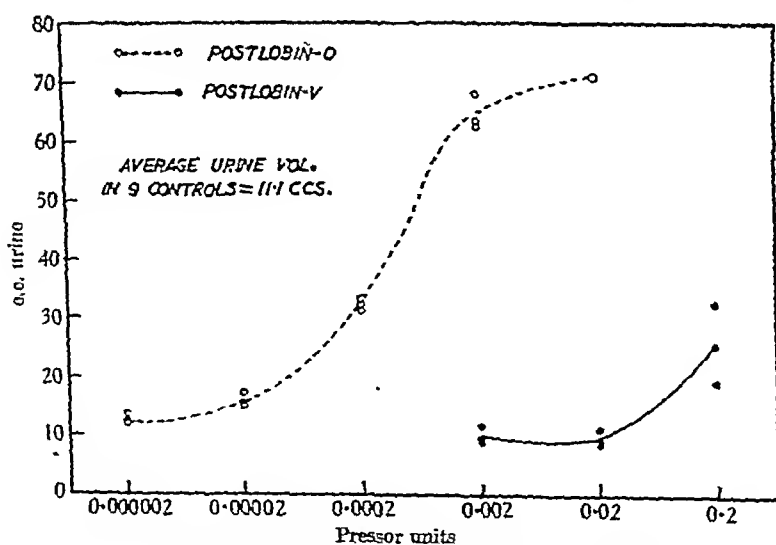


Fig. 2. In the case of postlobin-O, the doses represented denote amounts of contaminating pressor hormone.

Fig. 2 shows that the diuretic action of the two extracts is not proportional to pressor activity and that the diuretic action of postlobin-O is much greater than can be explained by contamination with pressor



hormone. Fig. 3 appears to show that increased water excretion postlobin-V is amply accounted for by its contamination with oxytocic activity. This is unlikely for several reasons. First, Exps. 6 and 7, shown in Fig. 1, render it improbable that diuretic action of the oxytocic contaminant of postlobin-V could show itself in the presence of the great excess of pressor hormone. Secondly, Fig. 6 shows that there is a difference between the courses of the diuresis of the two preparations. Thirdly, the consideration of the chloride excretions below reveals that

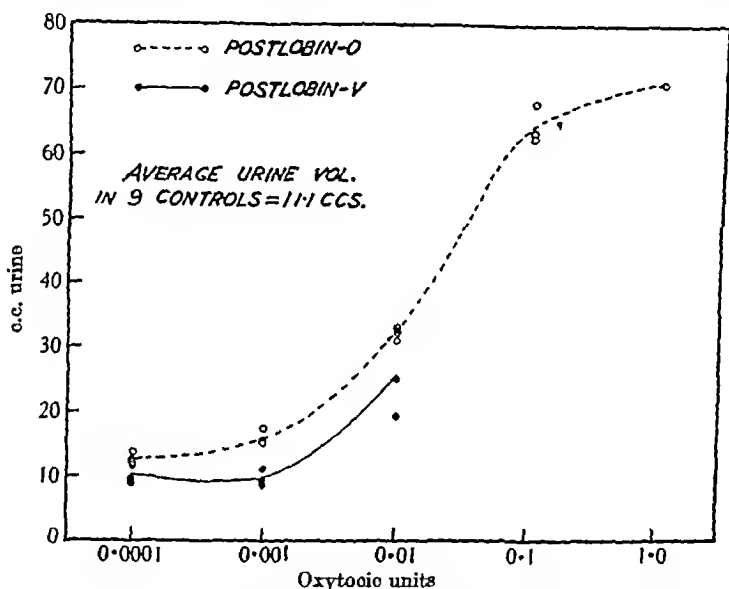


Fig. 3. In the case of postlobin-V the doses represented denote amounts of contaminating oxytocic hormone.

the increased water output after postlobin-V is probably a salt diuresis, as experiments of others have indicated.

The results in Fig. 4 show that the chloride excretion after postlobin-O is very much greater than is accounted for by its contamination with the pressor hormone. A definite increase in chloride excretion occurs after 0.00002 pressor unit postlobin-O, whereas no effect is produced by doses of postlobin-V smaller than 0.2 pressor unit. The difference in the shapes of the two curves suggests that the causes for the respective increases in chloride excretion may be different. Fig. 5 shows that the sudden rise in chlorine excretion on increasing the dose of postlobin-V from 0.02 to 0.2 pressor unit is somewhat more than can be accounted for by its oxytocic contamination. Table I shows that when the oxytocic prepara-

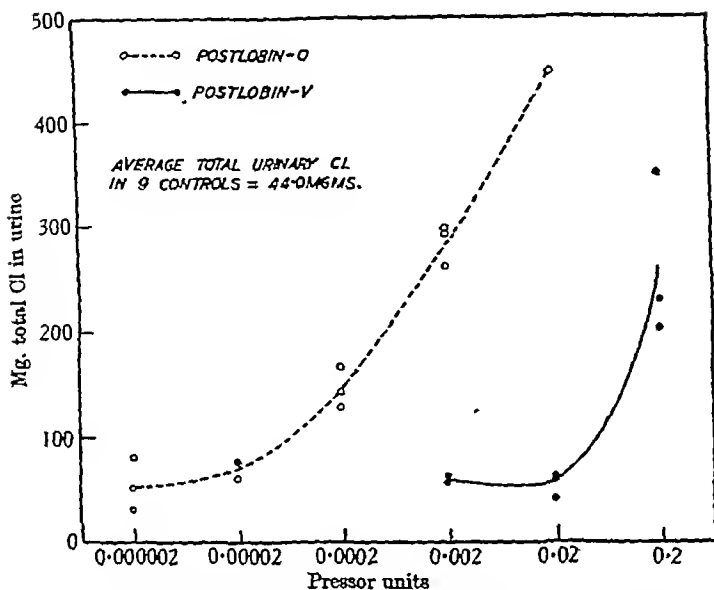


Fig. 4. In the case of postlobin-O, the doses represented denote amounts of contaminating pressor hormone.

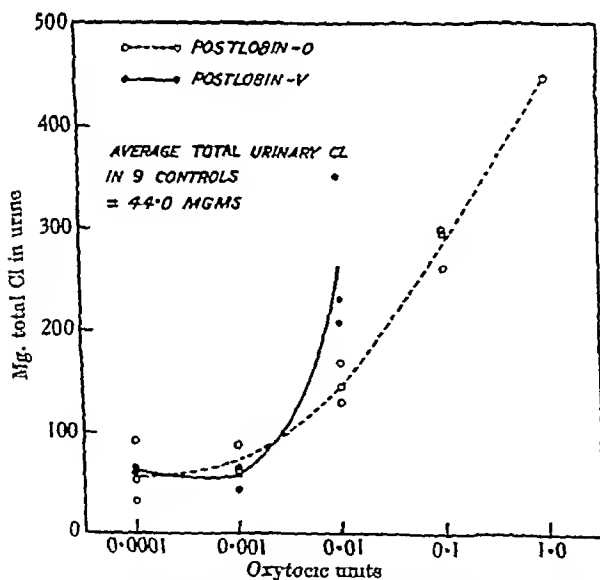


Fig. 5. In the case of postlobin-V, the doses represented denote amounts of contaminating oxytocic hormone.

tion is administered, though there is a large increase in the absolute quantity of chlorine excreted, there is little increase in the concentration until the dosage is high, and the increase may then be explained by the pressor contaminant. The pressor preparation, however, never causes a diuresis without having increased the chloride concentration, suggesting as stated above, that it is only a salt diuresis.

The data above makes it clear that postlobin-O is much more potent than postlobin-V in increasing water and chlorine excretion, and that this action of postlobin-O is not due to contaminating pressor hormone.

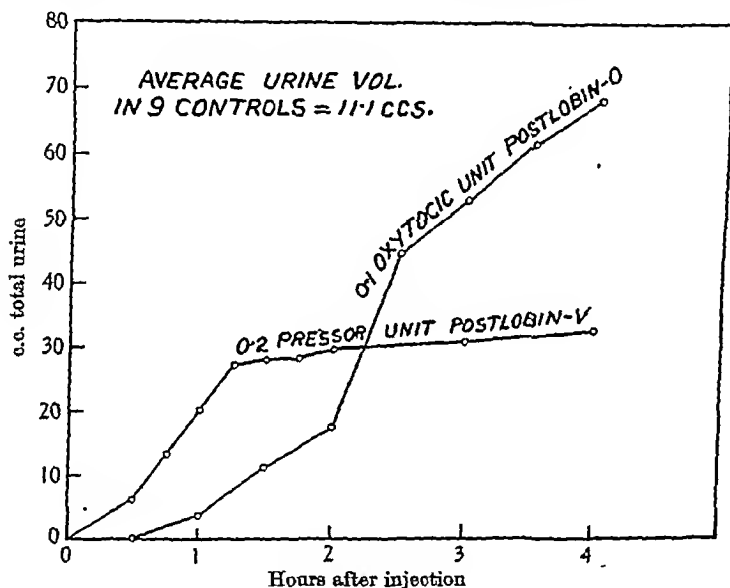


Fig. 6.

Qualitative and quantitative considerations also reveal that the increased output of water and chlorine after postlobin-V, although slight compared to that after postlobin-O, is not accounted for by oxytocic contamination, but is presumably due to the pressor hormone as others have suggested [Melville & Holman, 1934].

The pH of the urine rises after injection of either preparation (Table 1), the greater rise occurring after postlobin-O. The rise after postlobin-V may be due to oxytocic contamination.

During the experiments described above it was noted that there was a definite difference between the course of the diuresis after postlobin-O and that after postlobin-V. A comparison of two experiments illustrating this fact is shown in Fig. 6. After the pressor extract the greater

part of the diuresis occurred within the first hour, whereas after the oxytomic extract there was a latent period of about 2 hr. before the diuresis began. This again strongly suggests that the two types of diureses are produced by different principles and through different mechanisms.

During this work it was also noted that the latent period between the time of injection of postlobin-O and the onset of the diuresis varied considerably and that the larger the dose the longer was this latent period. This variation is illustrated by the experiments shown in Fig. 7.

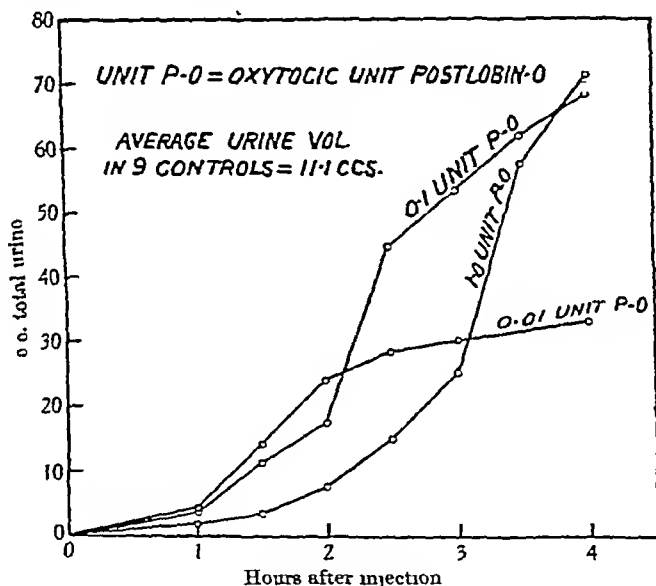


Fig. 7.

Their results suggested that the cause of the latent period might be the antidiuretic action of the pressor hormone contaminating postlobin-O. This suggestion was supported by the fact that small doses of pressor extract are capable of delaying oxytomic diuresis as shown in Fig. 8. This antagonism also demonstrates the essentially opposite actions of the two extracts.

Rats deprived of water for a few hours gave no diuretic response to administration of postlobin-O.

*Experiments with dogs.* Since all the work showing a diuretic action of oxytomic extracts has been performed on rats it seemed desirable to extend the study to another species. A note appended to the earlier paper [Fraser, 1937] stated that in performing several experiments on

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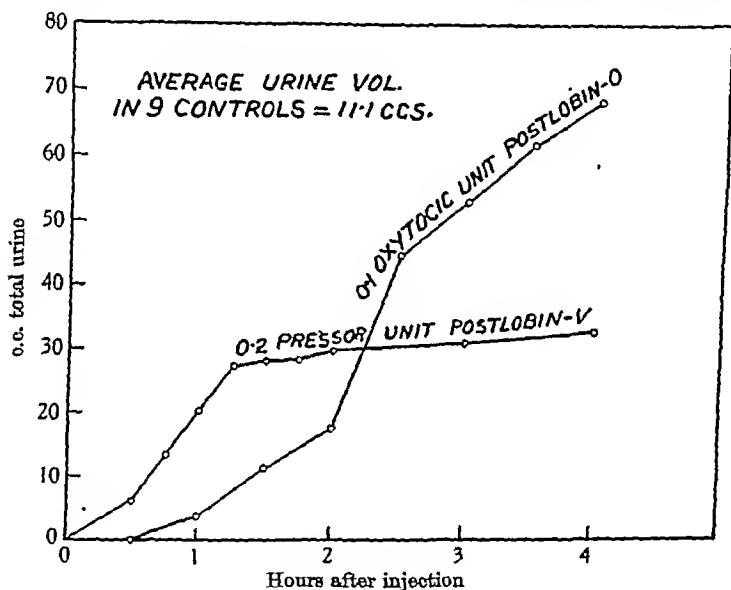


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Qualitative and quantitative considerations also reveal that the increased output of water and chlorine after postlobin-V, although slight compared to that after postlobin-O, is not accounted for by oxytocic contamination, but is presumably due to the pressor hormone as others have suggested [Melville & Holman, 1934].

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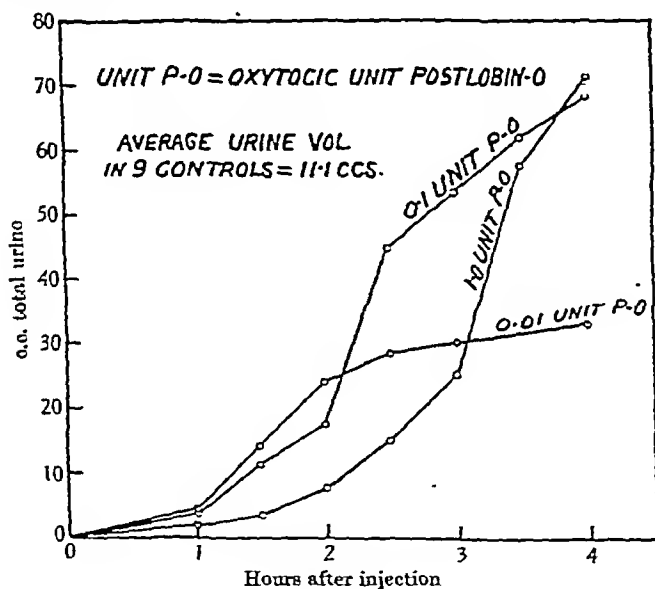


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dogs, there was no diuretic response to postlobin-O. Those injections were made intravenously. During the present work the first experiments with dogs were also done using this route, and the results were also doubtful. On resorting to the subcutaneous method, however, a definite response in dogs was obtained. Although this was less marked than in rats the time of onset and the duration of the diuresis was similar to that observed in rats.

Some of the dogs used were females provided with bladder fistulae. These dogs were trained to lie on a table in which a funnel was placed

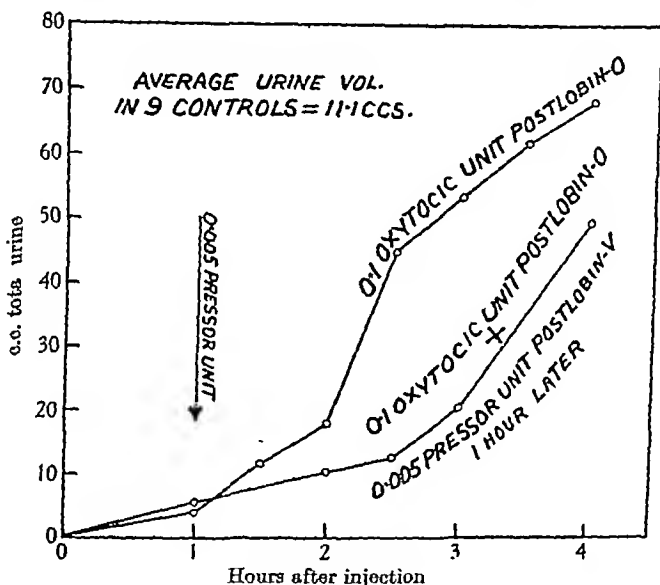


Fig. 8.

so that the urine was collected in graduated cylinders as it dropped from the ureteral orifices. Table 2 shows the results of one of the experiments. It will be noted that both total chlorine and chlorine concentration are increased. The effect on phosphorus is remarkable and was studied further.

An attempt was made to demonstrate the accelerating action of postlobin-O on water excretion from hydrated dogs. Several difficulties were encountered. Controls varied considerably from day to day in the same animal. The dogs also became capable of excreting water more and more rapidly, when they were being given large quantities of water frequently. On the whole the results were not very convincing. It is believed also that the pressor contamination in postlobin-O obliterates

the diuretic action to a greater extent in the dog than in the rat. The lower sensitivity of the dog to the diuretic action of postlobin-V, as demonstrated in non-hydrated dogs, would suggest this.

Daily injections of postlobin-O were found to produce no increase in thirst on dogs or rats.

TABLE 2. Bladder fistula dog, wt.=10.0 kg. No food since day before at noon.  
500 c.c. water at 5.00 p.m. day before, none afterward

Time	C.c. urine	Mg. total Cl	Mg. Cl per c.c.	Mg. total P
9.30- 9.45 a.m.	3.3	11.1	3.36	4.1
9.45-10.00	4.2	9.9	2.36	4.4
10.00	1 unit postlobin-O per kg. subcutaneously			
10.00-10.15	3.3	8.9	2.70	3.2
10.15-10.30	3.9	9.0	2.30	3.2
10.30-10.45	3.8	21.7	5.71	0.32
10.45-11.00	10.0	55.8	5.58	0.25
11.00-11.15	8.7	55.1	6.33	0.23
11.15-11.30	7.6	40.2	5.29	0.19
11.30-11.45	8.2	34.4	4.20	0.26
11.45-12.00 noon	13.0	34.8	2.68	0.25
12.00-12.15 p.m.	7.6	31.5	4.15	1.15
12.15-12.30	7.2	33.4	4.64	2.66
2.30- 2.45	3.2	6.9	2.16	2.14
2.45- 3.00	3.2	8.0	2.50	2.56

### *Effect of pituitary extracts on phosphorus excretion*

Dogs with bladder fistulae were used in this study, the technique being similar to that described in the preceding section.

The results on two dogs are summarized in Tables 3 and 4. Exp. 2 shows that a dose as small as 0.001 unit of postlobin-O lowers the excretion of phosphorus. As the dose is increased this effect becomes more marked as is evident in Exps. 3-5. But when a dose of 10 units is injected there is no reduction in the phosphorus output. This negative result is probably caused by the antagonizing action of the pressor hormone which contaminates the oxytomic extract. This phosphorus-raising action of the pressor hormone is seen in Exps. 7 and 8—an action noted by Stehle & Bourne [1925]. The second animal was less sensitive to the action. Exps. 13-16 show that small doses of the pressor hormone have no effect on phosphorus excretion, and therefore the depression of phosphorus excretion by the oxytomic extract is not due to its contamination with the pressor hormone.

Experiments conducted over a longer period show that this action of postlobin-O lasts from 1 to 3 hr.



TABLE 3. Bladder fistula dog, wt. 9.8 kg. Food: 200 g. Purina Fox Chow daily at 4.00 p.m. No food in cage after 5.00 p.m. Water: 250 c.c. water by stomach tube at 9.30 a.m. on day of experiment. Drinking water removed at same time. Experiments were begun about 2.00 p.m.

Min. after begin. exp.	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 5		Exp. 6		Exp. 7		Exp. 8	
0-15	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.
15-30	7.8	0.178	1.8	0.250	3.4	0.184	4.3	0.230	4.0	0.338	10.2	0.485	4.3	0.186	4.2	0.172
30	9.1	0.097	2.1	0.190	3.8	0.112	4.3	0.115	5.6	0.179	12.5	0.577	4.6	0.194	3.9	0.063
	Control (saline)		0.001 unit P-O		0.01 unit P-O		0.1 unit P-O		1.0 unit P-O		10 units P-O		1.0 unit P-V		10 units P-V	
30-45	8.6	0.150	2.2	0.087	2.0	0.073	1.4	0.050	1.4	0.089	5.4	0.527	1.3	0.077	0.6	0.008
45-60	6.2	0.157	3.2	0.062	4.3	0.019	4.1	0.018	3.6	0.010	11.3	0.577	3.5	0.440	1.3	0.269
60-75	6.8	0.130	4.1	0.057	4.2	0.013	6.1	0.013	11.5	0.011	14.5	0.421	6.1	0.279	5.8	0.643

TABLE 4. Bladder fistula dog, wt. 8.0 kg. Food: 200 g. Purina Fox Chow daily at 11.00 a.m. No food in cage after 5.00 p.m. Water: 500 c.c. water by stomach tube at 5.00 p.m. on day before experiment. Drinking water removed from cage at same time. Experiments were begun about 9.30 a.m.

Min. after begin. exp.	Exp. 9		Exp. 10		Exp. 11		Exp. 12		Exp. 13		Exp. 14		Exp. 15		Exp. 16	
0-15	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.	C.c. urine	Mg. P/min.
15-30	1.7	0.137	1.3	0.114	6.0	0.121	1.9	0.152	7.1	0.355	3.5	0.161	3.7	0.148	4.1	0.145
30	1.4	0.081	1.5	0.084	4.5	0.098	4.3	0.267	4.8	0.407	4.2	0.151	7.5	0.195	12.5	0.283
	0.01 unit P-O		0.1 unit P-O		1.0 unit P-O		10 units P-O		0.0002 unit P-V		0.002 unit P-V		0.02 unit P-V		0.2 unit P-V	
45	1.0	0.050	1.8	0.101	5.3	0.125	1.2	0.079	5.4	0.411	0.9	0.126	1.8	0.183	1.9	0.197
60	1.1	0.040	2.7	0.017	9.5	0.023	4.1	0.052	4.4	0.413	2.3	0.157	8.2	0.150	15.0	0.370
75	1.4	0.047	2.0	0.012	12.9	0.025	8.4	0.009	5.7	0.363	2.7	0.119	12.0	0.136	20.0	0.413

## DISCUSSION

There is good reason to believe that the strong diuretic action exerted by the extracts of the posterior lobe of the pituitary gland is produced by the oxytomic hormone. This was suggested in the earlier paper [Fraser, 1937] because it was found that the two oxytomic preparations, postlobin-O and pitocin, which are prepared in different ways, have approximately equal diuretic actions when compared on the basis of oxytomic activity. The high sensitivity of hydrated rats to these preparations lends support to this idea—0.0001 oxytomic unit of postlobin-O per 100 g. had a definite accelerating action on water excretion. This quantity of postlobin-O has a weight of only 0.0000004 mg. It seems very improbable that the diuretic action is due to a substance which contaminates this quantity of substance.

One is unable to conclude whether the action of postlobin-O on urine secretion is directly on water excretion or on salt excretion. The accelerating action on water excretion from hydrated rats shows that it is capable of producing a very dilute diuretic urine. In non-hydrated rats, however, the action on chlorine excretion is as great as that on water excretion. The fact that slight dehydration destroys the urinary response suggests that the action on water excretion is essentially one on excess body water.

Since the oxytomic hormone has a hyperglycemic action in dogs [Holman & Ellsworth, 1935] it is suggested that the action of postlobin-O on phosphorus excretion may be associated with carbohydrate metabolism. This action on phosphorus excretion is effected by doses even smaller than is the diuretic action. In Table 3 it is seen that 0.001 unit in a dog weighing about 10 kg. had a definite action. This dose is equivalent to 0.0000004 mg./kg.

The minimal doses required to elicit the diuretic action and the action on phosphorus excretion are 100 and 1000 times smaller respectively than the dose required to contract the isolated uterus of the guinea-pig immersed in 100 c.c. of Locke solution. It will be observed, too, that the diuretic action is produced by oxytomic doses as small as are the pressor doses which elicit minimal antidiuretic effects. The high sensitivity to these actions of the oxytomic extract warrants consideration of the possibility of their physiological significance in maintaining water balance. Until now only the antidiuretic action has been regarded as a physiological function of the posterior-pituitary gland. It now seems easy to imagine that body water balance may be maintained physiologically by

an interplay between the diuretic and the antidiuretic actions of the oxytocic and pressor hormones respectively.

The diuretic action of the pressor extract appears to be elicited only by doses which would have drastic circulatory effects. It seems probable that this salt diuresis is a result of the toxic circulatory action of these large pressor doses. Since the oxytocic extract is extremely active in increasing chlorine excretion it is likely that the oxytocic hormone is responsible for increases in chloride excretion observed by many workers after administration of small doses of pituitary extract.

#### SUMMARY AND CONCLUSIONS

1. The diuretic action of a preparation of the oxytocic hormone (postlobin-O) has been studied and compared with that of a preparation of the pressor hormone (postlobin-V).

2. Postlobin-O is much more active than postlobin-V in increasing chlorine and water excretion in non-hydrated rats.

3. Postlobin-V antagonizes the diuretic action of postlobin-O in hydrated and non-hydrated rats.

4. Postlobin-O is extremely active in decreasing phosphorus excretion.

5. Considerations of the quality and magnitude of these urinary actions of postlobin-O show that they are not due to the pressor hormone, but are very probably properties of the oxytocic hormone. Similarly, the diuretic action of postlobin-V is shown to be due to a substance other than the oxytocic hormone, in agreement with previous workers who believe the action is a property of the pressor hormone..

6. These urinary actions of postlobin-O are elicited by doses as small as those of postlobin-V necessary to produce antidiuretic action, and smaller than doses of postlobin-O required to exert oxytocic action. Attention is therefore drawn to the possible physiological significance of these urinary actions of postlobin-O.

The writer is indebted to Prof. R. L. Stehle for valuable advice and criticism during this work.

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## THE EFFECT OF HISTIDINE ON HISTAMINE SHOCK

BY O. G. EDHOLM

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THE fate and effect of histidine in the body are of interest, as this substance has been used in the treatment of gastric ulcers. The presence of an enzyme, histidine decarboxylase, in the kidney of certain species has been demonstrated by Werle & Herrmann [1937]. This enzyme converts histidine into histamine. Zipf & Gebauer [1937] could not confirm these findings, but Werle & Heitzer [1938] and Werle & Krautzun [1938] have brought forward further evidence in support of these views. The presence of this enzyme in the kidney has been confirmed by Holtz, Heise & Spreyer [1938], and by Parrot [1939].

Indirect evidence of the transformation of histidine into histamine is given by Bloch & Pinösh [1936], who found that the histamine content of the lungs was increased following the injection of histidine. Their results have been criticized by Mackay [1938], who showed that the increased histamine content of the lungs came within the normal range of variation. Mackay [1938] also showed that the injection of histidine into animals did not alter their reaction to histamine.

The effect of histidine on the isolated uterus and intestine was investigated by Mackay [1938] and by Halpern [1939]. They found that the mobility of both intestine and uterus was reduced by the addition of histidine to the perfusate, and that histidine antagonized the action of histamine on both uterus and intestine.

Both Bloch & Pinösh [1936] and Mackay [1938] examined the acute effects of histidine, the animals being tested within five hours of the injection. In clinical work daily injections of histidine are given for a period of four weeks. Since there is evidence that histidine can be converted into histamine in the body, it was considered that the effects of daily injections of histidine into animals for a similar period should be examined.

## METHODS

Forty-one cats were used in these experiments. All of these received daily subcutaneous injections for 28 days. Fourteen received 1 c.c. of a 4% solution of *l*-histidine monochloride (larostidine), five received 1 mg. histamine in 1 c.c. water, and the rest (twenty-two animals) acted as controls having a daily injection of 1 c.c. water. At the end of the 28 days of injections the animals were anaesthetized with ether, followed by chloralose (0.065 g./kg. body weight) and the blood pressure recorded from a cannula inserted into the common carotid artery.

The vascular reactions to histamine injected intravenously were studied: first following the injection of 0.5 c.c. of a 1 in 100,000 solution of histamine, and then a shock dose of 3 mg. histamine.

In a second series of experiments, guinea pigs were used, thirteen animals in all. Five received daily subcutaneous injections of 5 c.c. 4% *l*-histidine monochloride, and the remaining eight received the same quantity of distilled water. The injections continued for 21 days. The animals were then killed, and the response of the isolated uterus to histamine was recorded. A double uterus bath was used, in one the horn from a histidine injected animal and in the other a horn from a control animal. The two baths were of equal capacity, 100 c.c., and Tyrode solution was used. The experiment was completed by using the second horns from the two animals and comparing the results obtained.

## RESULTS

The two groups of animals, those injected with histidine and the controls, were selected to provide the same sex distribution and the same average weight (Table 1). The injection of histidine did not have any

TABLE 1. The effect of histidine injections on cats

	Histidine group	Histamine group	Controls
Number of animals	14	5	22
	8 ♂ 6 ♀	2 ♂ 3 ♀	12 ♂ 10 ♀
Average weight in kg.:			
Initial (before injections)	2.66	2.70	2.62
Final (after injections of histidine)	2.52	2.59	2.52
Average blood pressure in mm. Hg	143.0	142.0	142.5
% fall of blood pressure following injection of 5 µg. histamine	39 ± 2.4	45 ± 1.5	47 ± 1.7
Deaths after injection of 3 mg. histamine	0	2	8
Degree of blood pressure recovery 2 hr. after injection of 3 mg. histamine	96% ± 9.5	76% ± 8.5	67% ± 8.3

effect on weight as compared with the controls. The systemic blood pressure, recorded when the animals were anaesthetized, was also similar in the two groups.

The fall of blood pressure following the intravenous injection of 0.5 c.c. histamine 1 in 100,000 was significantly smaller in the histidine injected animals than in the controls (Table 1). After recovery from this injection was complete, a shock dose of 3 mg. of histamine was injected intravenously. All of the histidine-injected animals recovered, but eight of the controls died. By recovery is meant the survival of the animal for three hours following the injection. At the end of three hours, the average blood pressure level expressed as a percentage of the original level before injection was considerably higher in the histidine animals than in the controls ( $96\% \pm 9.5$  and  $67\% \pm 8.3$  respectively). These results show a statistically highly significant difference in the reaction of the two groups.

The reactions of the group of animals, previously injected with histamine, did not show any significant difference from those of the controls.

The uteri of the histidine-injected guinea-pigs were less responsive to histamine than those of the control group. The sensitivity of the uterus from different animals varied considerably. In the control group, three out of eight gave a measurable contraction on the addition of 1 c.c. 1 in  $10^7$  histamine, one of the five injected animals responded. Using a stronger solution of histamine, all of the control group responded to 0.5 c.c. 1 in  $10^6$  histamine, but only three of the five injected. The remaining two only contracted when 0.5 c.c. 1 in  $10^5$  histamine was added to the bath. The two horns from the same animal gave very similar responses, and are therefore counted as one.

Applying the  $\chi^2$  test, there is a significant difference between the response of the two groups of uteri to a concentration of 0.5 c.c. 1 in  $10^6$  histamine.

### DISCUSSION

These experiments show that pre-treatment with histidine definitely diminishes the sensitivity of the vascular system of the cat to histamine. The dose of histidine employed was considerably larger than those used in man, weight for weight. In clinical work, 5 c.c. of the 4% solution is employed. No ill effects were observed during the four weeks of injection.

Histamine itself in these experiments, when it was used as a desensitizing agent, was without effect. However, the dose used was small, 1 mg. as compared with 40 mg. of histidine. Karady [1936] has shown that larger doses of histamine can change the vascular reactions of cats, converting the depressor action of histamine into a pressor. Histamine

desensitization by histamine has also been found in the guinea-pig, as shown by the insensitivity of the bronchi of injected animals as compared with controls [Sutherland, 1938]. In these experiments, such large doses of histamine were used that the animals lost weight considerably. In the dog, Jacobs & Masson [1936] found that continuous injection of histamine for 62 days produced less and less toxic effects.

It is probable from these reports, in spite of the negative results obtained in the present research, that histamine in sufficient quantities can desensitize animals.

It has already been shown that histidine does not produce a rapid change in the vascular response of the cat to histamine [Mackay, 1938]. In the anaesthetized animal, the intravenous injection of histidine had no effect on the fall of blood pressure produced by histamine.

Werle and his associates [1937, 1938] have produced abundant evidence that histidine can be converted into histamine.

It is probable that the results described in this paper are due to the conversion of part of the histidine injected into histamine; this histamine produces a desensitizing effect, as shown by the decreased fall of blood pressure when histamine is injected.

The reduction of the sensitivity of the isolated uterus of histidine-injected guinea-pigs confirms the effect on the cat. Mackay [1938] and Halpern [1939] have both shown that histidine added direct to the uterus bath reduces the mobility of the uterus and its sensitivity both to histamine and pituitrin.

The advantage of histidine, as a desensitizing agent, lies in the lack of toxic effects such as are produced by histamine itself.

#### SUMMARY

The effect of histidine on the action of histamine has been examined in cats and guinea-pigs.

In cats, after twenty-eight daily subcutaneous injections of histidine, the depressor action of histamine on blood pressure was significantly diminished.

The sensitivity to histamine of the uterus of guinea-pigs similarly treated with histidine was also reduced.

Daily injections of histidine did not affect either the weight or the blood pressure of the cats.

I wish to thank Professor R. J. S. McDowall for his help and advice. The experimental work was carried out during the tenure of a Boveri Research Scholarship. Messrs Hoffmann-La Roche provided generous supplies of histidine (larostidine).



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FACTORS AFFECTING BICARBONATE CONTENT,  
FREE CO<sub>2</sub> AND pH OF URINE

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BICARBONATE content, pH, and free CO<sub>2</sub> of urine have been the subject of investigation on numerous occasions. Unfortunately, most of the investigators have been interested in either the bicarbonate or the free CO<sub>2</sub>, but not their mutual behaviour. This has meant that there has been little attempt to correlate the data already in existence on the subject.

Most of the investigators have directed their attention to the free CO<sub>2</sub> of urine; the bibliography and the findings to 1934 are to be found in Van Slyke's paper [Sendroy, Seelig & Van Slyke, 1934]. There appears to have been only one paper on the subject since then [Sarre, 1937].

The values reported by Mainzer & Bruhn [1931] for free CO<sub>2</sub> range from 13 to 242 mm. Hg. Sendroy *et al.* [1934] did not obtain values below 40 mm. Hg, but one must bear in mind that his collection periods ranged from 1 to 10 hr. Periods of this length make it possible that the extreme variations in value are cut out.

The present work started as a study of the differences in pH between the denervated and innervated kidney of the same animal. The differences found led to an examination of bicarbonate content and free CO<sub>2</sub> and some of the factors which affect them.

## METHODS

The work reported here has been carried out on dogs and cats. In the case of the dog, the urine was collected by ureteric cannulae emptying directly into a glass vessel under paraffin. In the cat, ureteric cannulation is difficult, and it was found necessary to insert a cannula through the urethra into the bladder. A ligature was tied round the bladder just above the trigone, which stopped the urine collecting in the bladder, and therefore enabled the urine to be collected as in the dog.

Estimations of pH were done by capillary glass electrode, at first on collected samples; later, in order to avoid any possible loss of CO<sub>2</sub>,

the sample was taken by connecting the capillary electrode to the collecting tube. This procedure has the added advantage of avoiding the contamination of the electrode by the paraffin. The electrode was restandardized frequently, usually after each estimation, correction being made for drift when present and significant.

CO<sub>2</sub> analyses were done on the Van Slyke manometric apparatus. Two readings were taken on each sample under the same conditions. Free CO<sub>2</sub> and bicarbonate were then obtained from the nomogram for urine of Sendroy *et al.* [1934], or where the values lay outside its limits, from the Henderson-Hasselbach equation with the appropriate values for urine [Sendroy *et al.* 1934]. No attempt was made to test the validity of the method, as it was felt that without determination of individual values for  $\alpha$  and  $pK_1$ , such an attempt might well be misleading. All the animals, except the decerebrate cats, were anaesthetized with nembutal. The cats had both kidneys denervated; four of the dogs had one kidney denervated. A hydrating dose of 50–100 c.c. 0.9 % saline was administered intravenously to all animals on completion of operation.

## RESULTS

(1) Effect of operative procedure and anaesthetization. In sixteen cats, the pH of the bladder urine, removed immediately after anaesthetization, was in no case higher than 6.3. The first samples obtained on completion of operation were already alkaline or moved steadily upwards to between pH 7.2 or 8.05. In the dogs, after anaesthetization, the urine was not always alkaline; the range is perhaps best seen in the figures.

(2) In eight cats decerebrated without removal of pituitary, under ether, the urine was consistently acid, pH 5.6–6.0, both during the recovery period and subsequently.

(3) The high pH's of (1) may be due to (a) hydrating dose of saline, (b) laparotomy and handling viscera during denervation and cannulation, (c) anaesthesia. In the decerebrate cats in two instances, the hydrating dose of saline caused a slight rise of pH with recovery.

(4) In the dog, there is a difference in pH and bicarbonate content between the normal and denervated kidney, and also between the two normal kidneys. The difference in pH may be as great as one pH unit, or be insignificant, i.e. lie in the third decimal place (Figs. 1, 4).

(5) In the cats (denervated kidneys) the highest CO<sub>2</sub> tension observed was 150 mm. Hg; in the dogs (denervated kidneys) 310 mm. Hg. In the cats, the maximum bicarbonate content observed was 186 mM./l.; in the dogs (denervated kidney) 370 mM./l.

(6) In one dog and three cats, cutting down the blood supply by a clamp on the renal artery, or on the aorta in the cats, resulted in a further

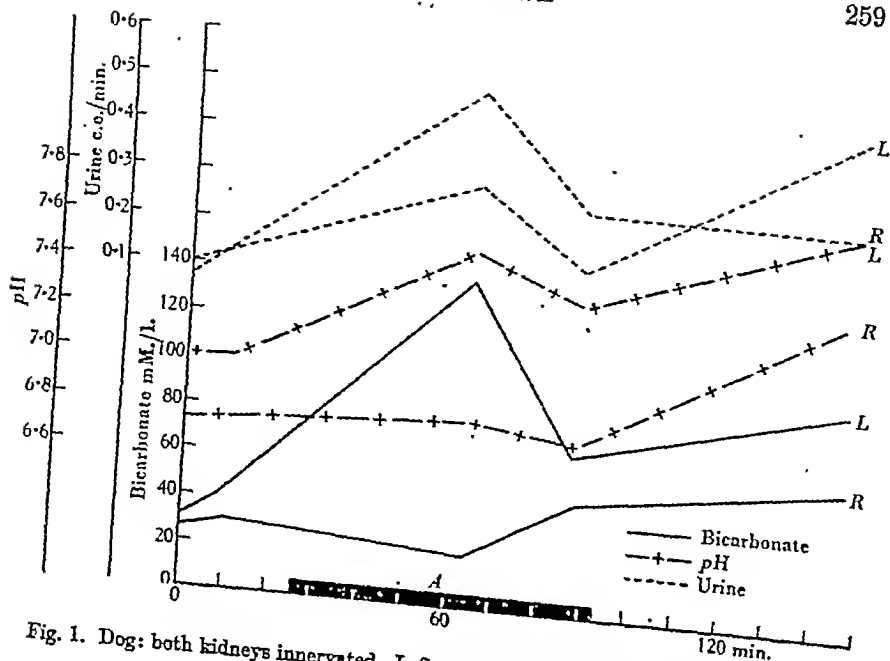


Fig. 1. Dog: both kidneys innervated. Left renal artery partially clamped during A.

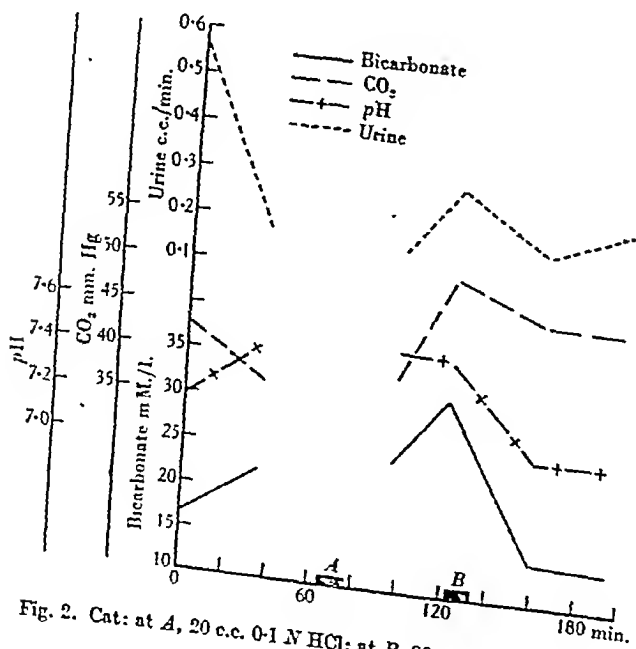


Fig. 2. Cat: at A, 20 c.c. 0.1 N HCl; at B, 20 c.c. 0.1 N HCl.

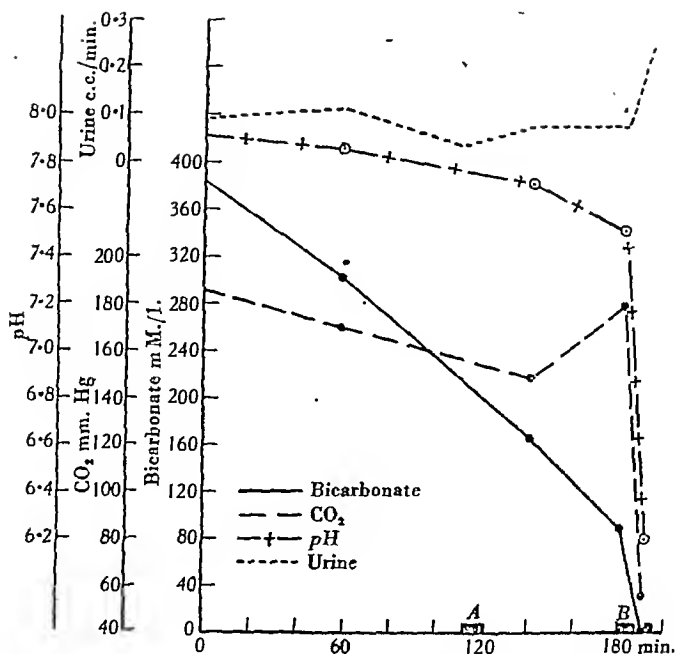


Fig. 3. Dog: innervated kidney. At A, 20 c.c. 0.1 *N* lactic acid; at B, 2 g. sodium sulphate in 50 c.c. saline.

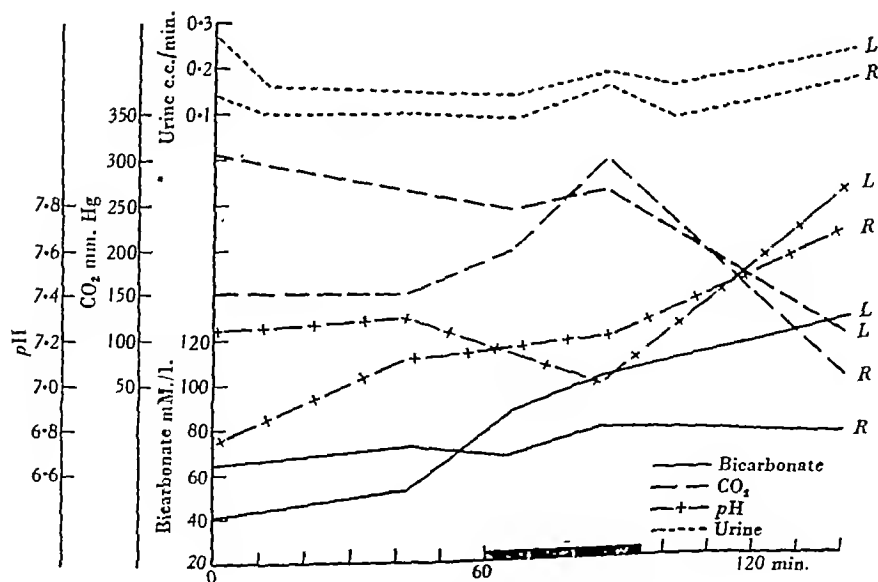


Fig. 4. Dog: left kidney denervated. At A, 5% CO<sub>2</sub>, 95% O<sub>2</sub>.

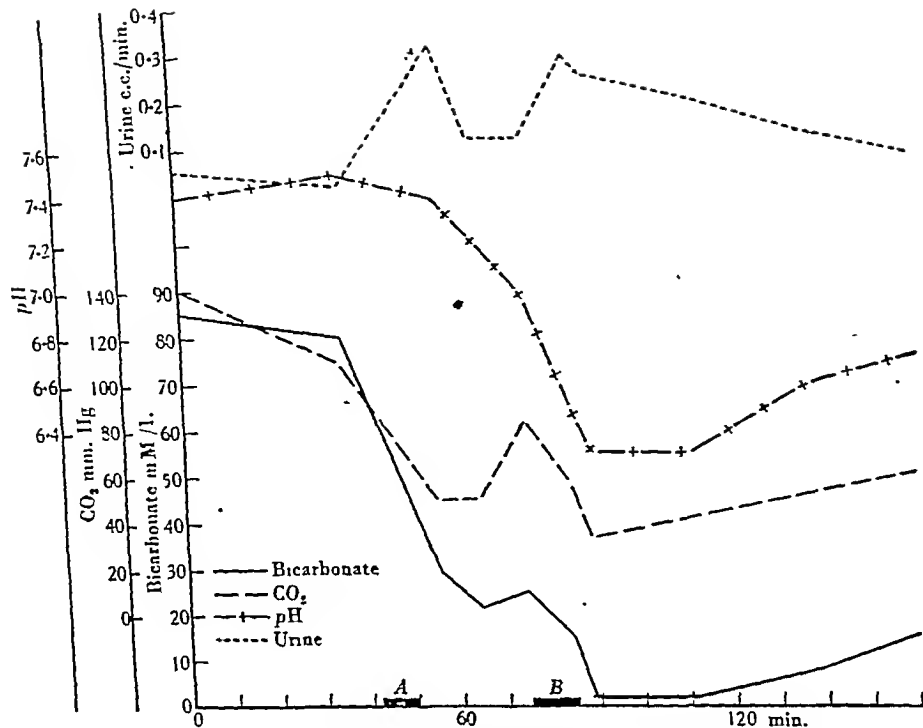


Fig. 5. Cat: at A, 1 g. urea in 26 c.c. normal saline; at B, 1 g. sodium sulphate in 25 c.c. normal saline.

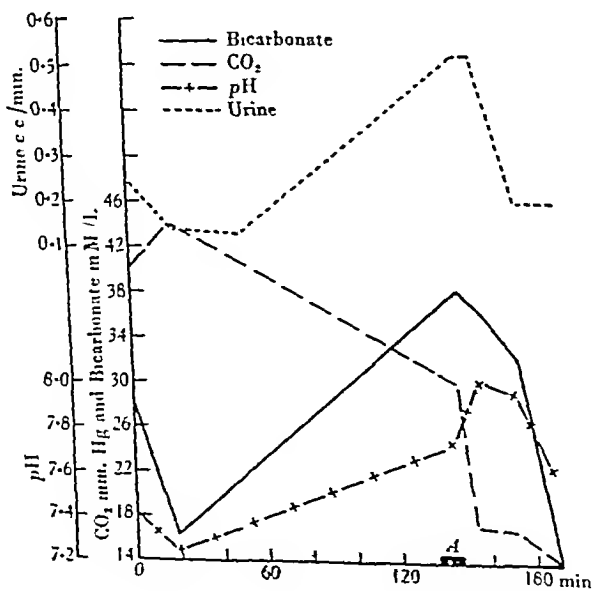


Fig. 6. Cat: at A, 1 g. sodium bicarbonate in 25 c.c. saline.

rise of bicarbonate in all cases, and a rise in  $pH$ , free  $CO_2$  tension or both (Fig. 1).

(7) In five animals, intravenous injection of 10–20 c.c. decinormal hydrochloric or lactic acid, or administration of 5%  $CO_2$ , 95%  $O_2$  led to little or no change in  $pH$  or bicarbonate content of urine. Further amounts of acid eventually caused a fall in bicarbonate and  $pH$  (Figs. 2–4).

(8) In marked contrast, in eight animals, intravenous administration of 1–2 g. sodium sulphate or urea dissolved in 25–50 c.c. saline, led to an immediate and rapid fall in  $pH$ , bicarbonate and free  $CO_2$ . The  $pH$  fell to a varying extent, usually to about  $pH$  6.2, with sodium sulphate (Figs. 3, 5). In one observation, disodium hydrogen phosphate had a similar effect to sulphate.

(9) In three cats, intravenous administration of 1–2 g. sodium bicarbonate in 25–50 c.c. saline, led to a marked fall in free  $CO_2$  (Fig. 6).

### DISCUSSION

The fact that the urine produced by the two kidneys differs in  $pH$  and bicarbonate content suggests that the level in urine is not determined primarily by the level in the blood. The experiments with acid bear out this point; not until the injection of acid causes what is presumably a large change in the level of one or other in the blood, is any great alteration in urine  $pH$  and bicarbonate found. Marshall & Crane [1923] studied the effect of cutting off the blood supply to the kidney for a short period. Comparison of the urines of the pre- and post-asphyxial periods showed that there was a distinct rise in  $pH$ , bicarbonate and free  $CO_2$  as the result of asphyxia. The rise in  $pH$  was never sufficient to give urines more alkaline than blood, except perhaps in one experiment where  $pH$  values are not given. Starling & Verney [1924] argued that these urines were in the process of moving up to the  $pH$  of plasma, and were essentially similar to those obtained in their experiments with cyanide. In so arguing, they neglected, or attached no importance to, the rise in free  $CO_2$  which should, of course, have fallen. Attention should perhaps be drawn to the fact that the rise in bicarbonate is not in proportion to the fall in urine flow.

The simplest explanation of the action of the diuretics would be that they increase blood flow, and so relieve anoxaemia. Such an explanation should be treated with caution. There does not appear to be an invariable increase of blood-flow during diuresis [Walker, Schmidt, Elam & Johnstone, 1937]. If it was entirely a matter of blood flow, one would expect that there would be a more marked difference in  $pH$  between

a denervated and innervated kidney. There is the further fact that removal of the clamp in the experiments of group (1) did not result in any marked drop in pH or bicarbonate.

The experiments on bicarbonate are included because it is felt that the drop in free  $\text{CO}_2$  places bicarbonate with the other diuretics. Examination of Mainzer's data [1929] shows that in man and dog, administration of large doses of bicarbonate also led to a fall in free  $\text{CO}_2$  in urine. It is of interest that Stieglitz [1924] showed that the tubule cells become acid after bicarbonate. It is true that McMaster & Elman [1928] could not confirm this with erythrolitmin, but found it might occur using neutral red as a tissue pH indicator *in vivo*. It may well be that the difference lies in the time elapsing after administration of bicarbonate and before sectioning the kidneys. Ellinger [1940] found that the tubule cells became acid after administration of urea and sodium bisulphate.

The values for free  $\text{CO}_2$  and bicarbonate for cat and dog should be compared with the figures for man, the highest  $\text{CO}_2$  tension reported being 242 mm. Hg [Mainzer & Bruhn, 1931], the highest bicarbonate content being 330 mM./l. [Davies, Haldane & Peskett, 1922]. The figures lend support to Peters's statement [1935] that the ability to maintain a high  $\text{CO}_2$  tension in the urine permits the excretion of a much higher concentration of bicarbonate at any given pH. This must naturally also apply to the pH at which the maximum bicarbonate content occurs.

There would appear to be three methods by which the kidney can excrete excess bicarbonate:

- (a) the maintenance of a high  $\text{CO}_2$  tension; this must not be too efficient or it will interfere with (b);
- (b) rise in pH;
- (c) diuresis.

The fact that the cat has for the most part to rely on the last two may well explain its inability to adapt to high altitudes [Dill, 1938].

#### SUMMARY

1. The urine from the two kidneys of the same animal differs in pH and bicarbonate content.
2. Most of the experimental animals had, as a result of anaesthetization and operation, an alkaline urine.
3. Cutting down the blood supply to the kidney results in a further rise in bicarbonate; pH, free  $\text{CO}_2$  or both may also rise.
4. Acid has not a very marked effect on bicarbonate, pH or free  $\text{CO}_2$ . Sodium sulphate led to a marked fall in all three; urea also led to a fall, although not to the same extent.



I wish to express my gratitude to Prof. H. P. Gilding for his valuable advice, to Dr D. B. Taylor, King's College, for the estimation of  $pH$ , to Mr G. Parkes for technical assistance, and to the University of Birmingham Research Committee for grants to meet expenses.

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## THE HAEMOLYTIC ACTION OF POTASSIUM SALTS

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STUDIES on the relative permeabilities of the cat erythrocyte to sodium and potassium have been described [Davson, 1939, 1940*a*, *b*]; the results were sufficiently interesting to warrant an extension of the work to other species of erythrocytes, and those of the dog were chosen since they have a very nearly identical cation constitution with those of the cat. With these cells, however, it was found that haemolysis was caused by placing them in isotonic solutions of potassium salts; the haemolytic effect varied with the anion of the salt, being most marked in thiocyanate and least in acetate, chloride having an intermediate effect. Pure solutions of potassium salts are well known to have a harmful influence on living tissues and cells, so that the haemolysis of the dog erythrocyte is perhaps not surprising; nevertheless, since the majority of the different species of erythrocyte do not haemolyse in potassium solutions, it was thought that the haemolysis might be caused by something else than a chemical damage to the membrane. The dog erythrocyte contains sodium as its chief cation constituent, so that in isotonic potassium chloride solution the distribution of cations is initially as follows:

Inside	Outside
0.170 <i>M</i> Na	0.165 <i>M</i> K
0.01 <i>M</i> K	

where concentrations are expressed as moles per kg. of water. (The concentration of cations is greater in the cells owing to the polyvalence of the haemoglobin ion.)

If the cell membrane is permeable to potassium, the latter will enter the cell, and if sodium either does not leak out at all, or does so at a rate less than that at which potassium penetrates, the increase in the osmotic pressure of the cell contents should cause the cell to swell and in time, as more potassium enters, to haemolyse. In the present paper evidence will be presented in support of this mechanism for the haemolytic action of potassium salts.

## METHODS

1.4 ml. lots of defibrinated whole blood, drawn by heart puncture, were measured with a syringe pipette into 10 ml. lots of saline, contained in 15 ml. centrifuge tubes which were immersed in a water bath at 25°. After appropriate intervals the suspensions were centrifuged and the amount of sodium or potassium in the packed cells was determined by methods described earlier [Davson, 1937, 1940a]. By comparing these values with those obtained on suitable controls, it is clear that any change in the average amount of cation per cell which takes place is measured independently of any change in volume the cells may undergo. The control value for the sodium content was obtained by an analysis of cells added to isotonic potassium chloride (to which a narcotic was added to prevent any escape of sodium) and immediately centrifuged down; that for potassium was obtained by an analysis of cells centrifuged from isotonic sodium chloride. When the cells, which have been centrifuged from potassium chloride solutions, are to be analysed for their potassium content, they are rapidly resuspended in isotonic sodium chloride and centrifuged again; in this way interstitial potassium is removed, and the escape of potassium from the cells into the isotonic sodium chloride solution was sufficiently slow to cause a negligible error.<sup>1</sup> The dilution of the whole blood being only about 1 : 8, the suspensions had sufficient buffering power to maintain a reasonably constant  $pH$  of  $7.7 \pm 0.05$ . The degree of haemolysis of the cell suspensions was determined by a colorimetric estimation of the concentration of haemoglobin in the supernatant fluid.

## RESULTS

If the haemolysis in potassium salt solutions is due to the penetration of potassium it follows that there should be a correlation between rate of haemolysis and permeability to potassium. In Fig. 1 the course of haemolysis with time is shown for various salts, and in Fig. 2 the course of penetration of potassium is shown under identical conditions;<sup>2</sup> the parallelism between the two processes is sufficiently striking. Cells from blood kept overnight show a remarkable increase in permeability to potassium; hence we may expect these cells to haemolyse more rapidly

<sup>1</sup> The absence of appreciable error is due to two causes. First, the difference of concentration between the inside and outside of the cell is high when the cell is in potassium chloride solution and comparatively low when it is transferred to sodium chloride solution; second, the permeability to potassium is very much lower when the cells are suspended in a medium of high Na : K ratio (cf. Fig. 9).

<sup>2</sup> Since the volume of the cells undergoes changes with changes in the cation contents, it is desirable to express the cation content in such a way that changes in the amount per cell are indicated, rather than changes in concentration; this is achieved by expressing the content in moles per litre of cells at their original volume.

than those from freshly drawn blood. In Fig. 3 the course of penetration of potassium with time in the two instances is shown and the points where haemolysis begins are shown with arrows, and once again the evidence confirms theory. As Fig. 4 shows, a narcotic such as *n*-butyl carbamate reduces the permeability of the dog erythrocyte to potassium, and hence it should decrease the rate of haemolysis; this point is also demonstrated in the figure, the time required for haemolysis to begin being prolonged

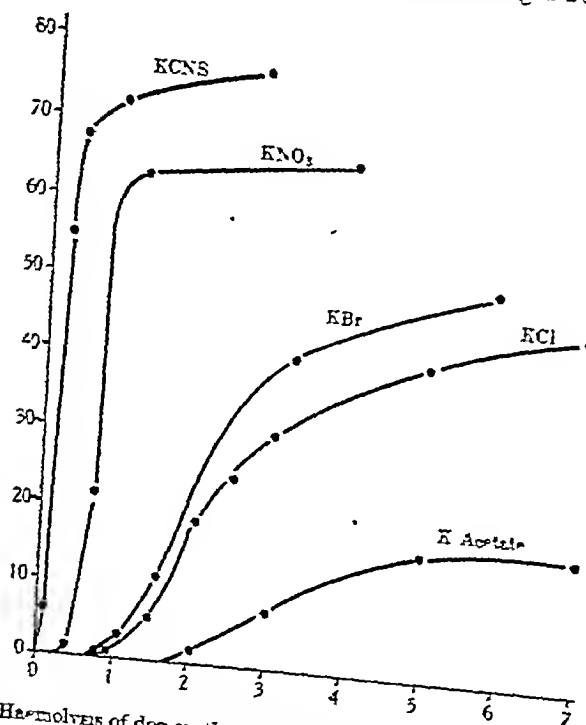


Fig. 1. Haemolysis of dog erythrocytes in isotonic solutions of potassium salts. Ordinates: percentage haemolysis. Abscissae: time in hours.

from about 1 to 8 hr. In Fig. 5 both the potassium and sodium concentrations of the cells are plotted against time of suspension in isotonic potassium chloride solution; in apparent contradiction to the theory of haemolysis suggested here the curves show that, although potassium penetrates the cells, sodium leaves them at almost exactly the same rate; at the points marked with arrows haemolysis had just begun and the figure shows that at this point the cells had gained 0.029 mol/l. of cells at their original volume and had lost 0.029 mol of sodium. Hence the average osmotic pressure of the cells had not been increased under these conditions and the theory of haemolysis apparently falls. Other objections to the theory may be deduced from Figs. 2-4, which show that the

## METHODS

1.4 ml. lots of defibrinated whole blood, drawn by heart puncture, were measured with a syringe pipette into 10 ml. lots of saline, contained in 15 ml. centrifuge tubes which were immersed in a water bath at 25°. After appropriate intervals the suspensions were centrifuged and the amount of sodium or potassium in the packed cells was determined by methods described earlier [Davson, 1937, 1940*a*]. By comparing these values with those obtained on suitable controls, it is clear that any change in the average amount of cation per cell which takes place is measured independently of any change in volume the cells may undergo. The control value for the sodium content was obtained by an analysis of cells added to isotonic potassium chloride (to which a narcotic was added to prevent any escape of sodium) and immediately centrifuged down; that for potassium was obtained by an analysis of cells centrifuged from isotonic sodium chloride. When the cells, which have been centrifuged from potassium chloride solutions, are to be analysed for their potassium content, they are rapidly resuspended in isotonic sodium chloride and centrifuged again; in this way interstitial potassium is removed, and the escape of potassium from the cells into the isotonic sodium chloride solution was sufficiently slow to cause a negligible error.<sup>1</sup> The dilution of the whole blood being only about 1 : 8, the suspensions had sufficient buffering power to maintain a reasonably constant pH of  $7.7 \pm 0.05$ . The degree of haemolysis of the cell suspensions was determined by a colorimetric estimation of the concentration of haemoglobin in the supernatant fluid.

## RESULTS

If the haemolysis in potassium salt solutions is due to the penetration of potassium it follows that there should be a correlation between rate of haemolysis and permeability to potassium. In Fig. 1 the course of haemolysis with time is shown for various salts, and in Fig. 2 the course of penetration of potassium is shown under identical conditions;<sup>2</sup> the parallelism between the two processes is sufficiently striking. Cells from blood kept overnight show a remarkable increase in permeability to potassium; hence we may expect these cells to haemolyse more rapidly

<sup>1</sup> The absence of appreciable error is due to two causes. First, the difference of concentration between the inside and outside of the cell is high when the cell is in potassium chloride solution and comparatively low when it is transferred to sodium chloride solution; second, the permeability to potassium is very much lower when the cells are suspended in a medium of high Na : K ratio (cf. Fig. 9).

<sup>2</sup> Since the volume of the cells undergoes changes with changes in the cation contents, it is desirable to express the cation content in such a way that changes in the amount per cell are indicated, rather than changes in concentration; this is achieved by expressing the content in moles per litre of cells at their original volume.

than those from freshly drawn blood. In Fig. 3 the course of penetration of potassium with time in the two instances is shown and the points where haemolysis begins are shown with arrows, and once again the evidence confirms theory. As Fig. 4 shows, a narcotic such as *n*-butyl carbamate reduces the permeability of the dog erythrocyte to potassium, and hence it should decrease the rate of haemolysis; this point is also demonstrated in the figure, the time required for haemolysis to begin being prolonged

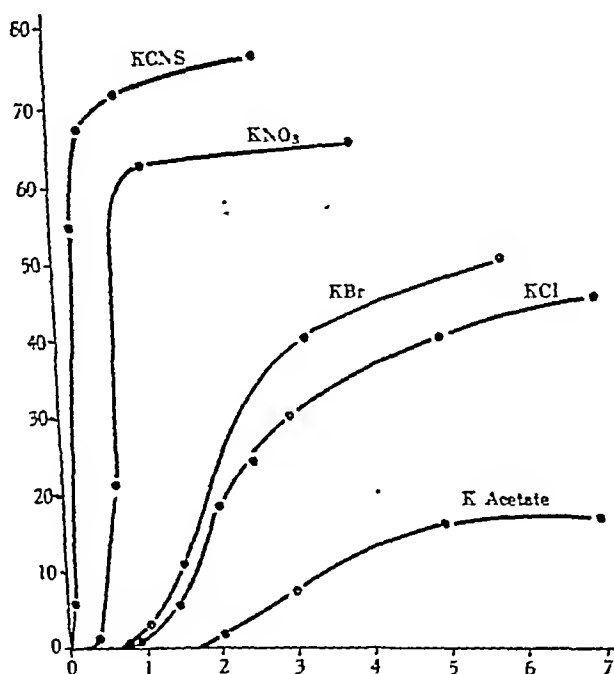


Fig. 1. Haemolysis of dog erythrocytes in isotonic solutions of potassium salts. Ordinate: percentage haemolysis. Abscissae: time in hours.

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for 1 hr. had increased from 0.0081 mol/l. of cells to 0.045 mol/l., removal of the top layer of cells reduced this value to 0.010 mol/l., indicating that practically all of the potassium gained by the cells had entered those making up the top layer; similarly, under the same conditions, it could be shown that the greater part of the sodium remaining

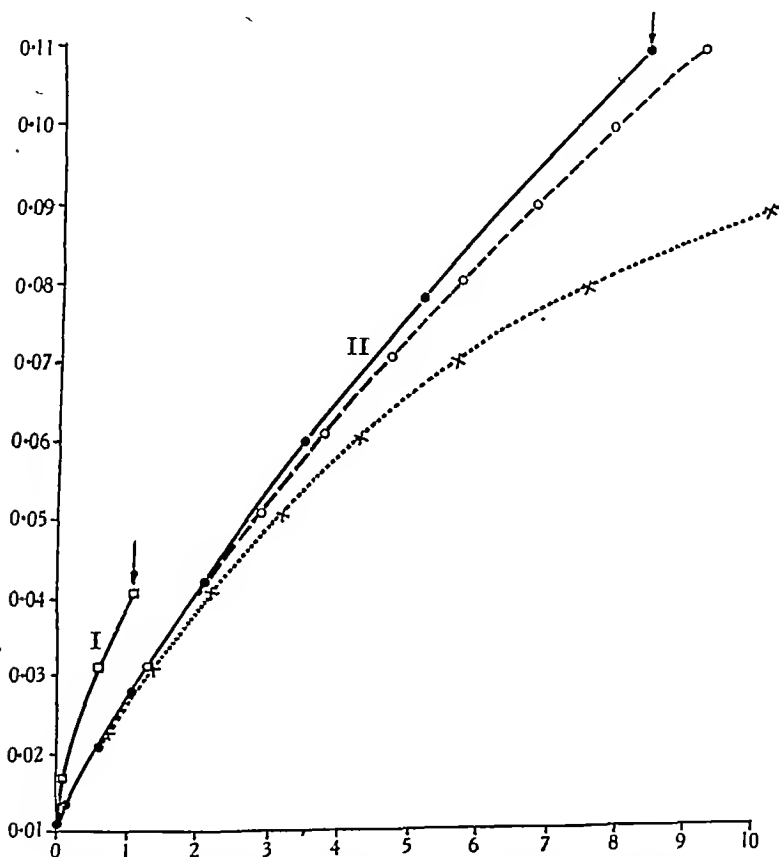


Fig. 4. Penetration of potassium into dog erythrocytes suspended in isotonic potassium chloride alone (curve I) and potassium chloride plus 0.6% *n*-butyl carbamate (curve II). Ordinates: potassium concentration of the cells in g. mol/l. of cells at their original volume. Abscissae: time in hours. O ..... O Curve for equation: (1). x ..... x Curve for equation: (2).

in the cells after 1 hr. was confined to the upper layer, i.e. that the lost sodium had migrated from the lower layer of shrunken cells. Thus the evidence strongly suggests that there is an abrupt discontinuity in the distribution of permeability characteristics among the cells of any given population of dog erythrocytes. The elucidation of the causes for this discontinuity is furthered by a study of the kinetics of the penetration



of potassium and of the escape of sodium, over longer periods of time than those used for Figs. 2, 3 and 5. In Figs. 6 and 7 the potassium and sodium concentrations are followed over periods of 5 and 7 hr. respectively; during these periods haemolysis of course takes place, and the percentage of haemolysis corresponding to the points on the curves is shown in parentheses. It must be appreciated that when haemolysis

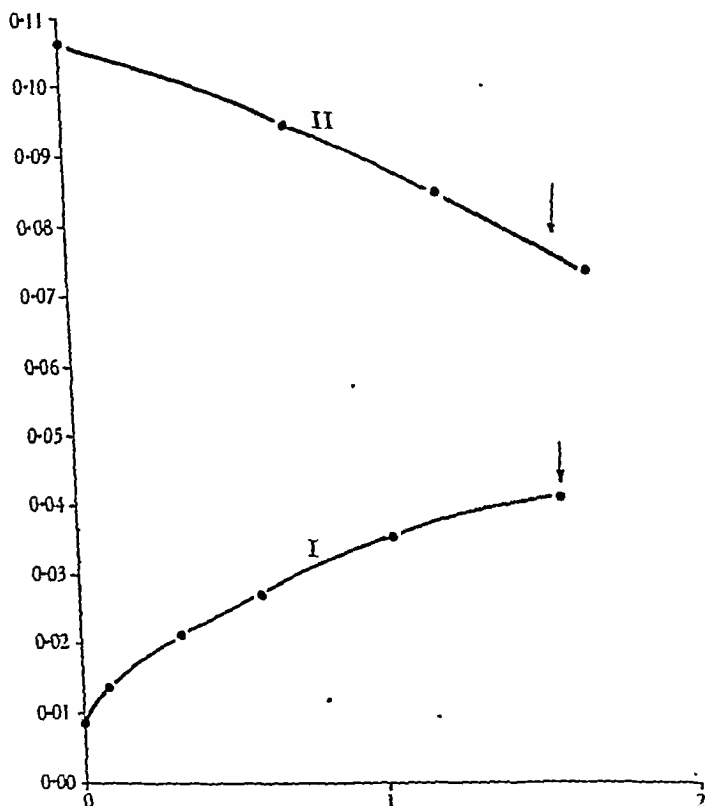


Fig. 5. Penetration of potassium into (curve I) and escape of sodium from (curve II) dog erythrocytes in isotonic potassium chloride. Ordinates: cell potassium or sodium concentration in g. mol/l. of cells at their original volume. Abscissae: time in hours.

takes place the number of cells recovered from the suspension is decreased, and hence the cation content is low by an amount equal to the amount of cation lost in the haemolysed cells; the curve of penetration will therefore, unless this effect is corrected for, show a falling off in the apparent rate which is not due to a slowing of penetration; in work on other red cells it has been found that a sufficiently good correction is obtained by simply adding on to the observed cation content the estimated amount of the loss due to haemolysis, assuming that those cells which

haemolysed contained the same amount per cell as did the average unhaemolysed cell. If this correction is made the curves in unbroken lines in Figs. 6 and 7 are obtained, and in Fig. 6 it is noted that there is an apparent decrease in the potassium concentration after the second hour; this is very unlikely since the concentration difference of potassium, although reduced, is still present after 2 hr., and it is much more reasonable

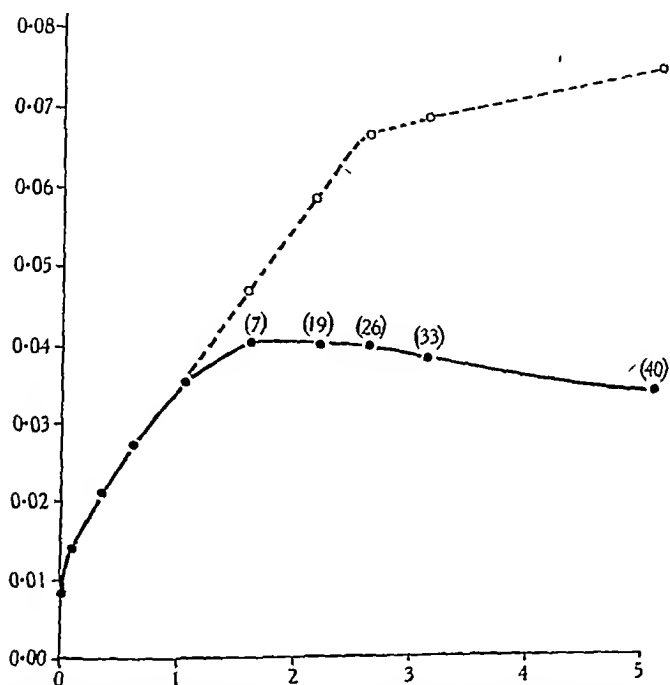


Fig. 6. Penetration of potassium into dog erythrocytes in isotonic potassium chloride. Figures in parentheses indicate the degree of haemolysis of the suspensions. Ordinates: cell potassium concentration in g. mol./l. of cells at their original volume. Abscissae: time in hours. For explanation of difference between broken and unbroken curves, see text.

to assume that the mode of correction for haemolysis is incorrect; if we assume that the haemolysed cells contained 0.11 mol of potassium per litre of cells, i.e. about the amount required to cause haemolysis assuming that this is a purely osmotic phenomenon, instead of the average value of about 0.04 and less, we obtain the curve in broken lines which does not show this anomaly of a decreasing cell potassium concentration;<sup>1</sup>

<sup>1</sup> When the degree of haemolysis is high, a further correction must be made to allow for the fact that the haemolysed cells are not taking up potassium; the curve in dotted lines has been corrected approximately for this factor too, and hence any falling off in the apparent rate of entry must be due to causes other than haemolysis, provided that the corrections are sufficiently accurate.

similarly, in Fig. 7 the apparent acceleration of the loss of sodium, when haemolysis begins, disappears when the haemolysis is corrected for by assuming that the haemolysed cells contained their original amount of sodium (0.115 mol).

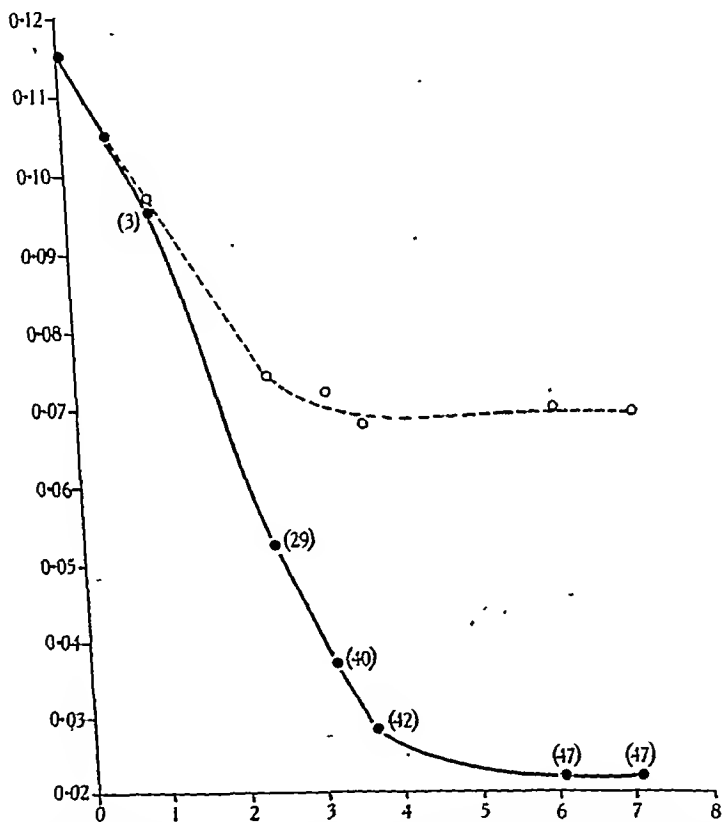


Fig. 7. Escape of sodium from dog erythrocytes in isotonic potassium chloride. Figures in parentheses indicate the degree of haemolysis of the suspensions. Ordinates: cell sodium concentration in g. mol/L of cells at their original volume. Abscissae: time in hours. For explanation of difference between broken and unbroken curves, see text.

If we turn attention now to the earlier portion of the curve for potassium penetration, before haemolysis has begun, we may note that there is a falling off in the rate of penetration with time; this is most noticeable in Figs. 2, 3 and 5, where the time scale is larger. Since penetration of potassium into the cell reduces the concentration difference, it must be expected that the rate should fall off; however, it is easy to show that the observed rate of falling off is far greater than any due to this effect; thus if we assume that potassium penetrates those cells which do not

lose sodium, penetration is followed by the entrance of water which dilutes the potassium and therefore minimizes the effect of a changed concentration difference; the equation for penetration in this case is

$$\frac{1}{2}S^2 + (p+q)S = kAqC_2t, \quad (1)$$

where  $S$  is the amount penetrated in time  $t$ ; all the parameters of this equation, except  $k$ , are known from the experimental arrangement, and it can be shown that, for the initial rate of penetration observed, during the first hour the deviation from linearity is negligible compared with that actually obtained.

If we assume that potassium enters cells which are losing sodium at the same rate, the equation becomes logarithmic of the form

$$\ln \frac{C_2V_0 - p}{C_2V_0 - p - S} = \frac{kAt}{V_0}, \quad (2)$$

and even in this case such a pronounced falling off in rate of penetration during the early stages should not be expected.

Equations (1) and (2) are derived as follows:

If  $S$  is the number of mols of potassium penetrating unit quantity of cells in time  $t$ , the differential equation for penetration is given by

$$\frac{dS}{dt} = kA(C_2 - C_1), \quad (3)$$

where  $C_2$  is the concentration of potassium in the suspension medium,  $C_1$  is the concentration of potassium in the cells,  $k$  is the permeability constant, and  $A$  is the area of unit quantity of cells.

Let  $p$  be the number of mols of potassium initially present in unit quantity of cells, and let  $V$  be the volume of water in this quantity at any time  $t$ .

$$\text{Then} \quad \frac{dS}{dt} = kA \left\{ \frac{C_2V - (p+S)}{V} \right\}. \quad (4)$$

$V$  is a function of  $S$  and  $t$  and must be eliminated before the equation can be integrated. The penetration of water into the cells is very rapid compared with the penetration of potassium, so that no error is introduced if it is assumed that there is osmotic equilibrium between the cells and their surroundings at any moment. If we let  $q$  be the quantity of osmotically active material (apart from potassium) in unit quantity of cells, expressed as mols of a univalent salt, the total concentration of osmotically active material in the cells is  $\frac{p+q+S}{V}$ . Hence

$$\frac{p+q+S}{V} = C_2. \quad (5)$$

Eliminating  $V$  from (4) and (5) gives

$$\frac{dS}{dt} = \frac{kAC_2q}{p+q+S}. \quad (6)$$

Since the volume of the suspension medium is large compared with that of the cells,  $C_2$  may be considered constant; moreover, the erythrocyte, by virtue of its biconcave shape, does not increase in area appreciably when swelling, so that  $A$  may also be considered constant. Equation (6) may therefore be integrated, giving

$$(p+q)S + \frac{S^2}{2} = kAqC_2t + \text{constant}. \quad (7)$$

When  $t = 0$   $S = 0$ , hence the constant of integration vanishes, and the equation for penetration is (1).

If sodium leaks out of the cells at approximately the same rate as that at which potassium enters, the volume of the cells remains constant,  $V_0$ , and in this case equation (4) becomes

$$\frac{dS}{dt} = kA \left\{ \frac{C_2 V_0 - (p + S)}{V_0} \right\}, \quad (8)$$

which may be integrated without further substitutions, giving

$$-\ln (C_2 V_0 - p - s) = \frac{kA t}{V_0} + \text{constant}. \quad (9)$$

Applying the same limiting condition as before, we get equation (2).

It should be noted that this treatment of penetration ignores any effects of diffusion potentials; as the permeabilities to sodium and potassium are found to be so nearly equal it is unlikely that these effects will be large, and they would rather modify the meaning of  $k$ , the permeability constant, than the form of the equations for penetration.

The falling off in rate may therefore be ascribed to some change in the cells that takes place during suspension in the potassium solution; the fact that the falling off was more pronounced in acetate than in chloride suggested that it was connected with the escape of sodium, since in acetate solution this escape is about twice as rapid as in chloride. The escape of sodium tends to make the cells shrink, so that if it is assumed that shrinkage of the cells reduces their permeability to potassium, ( $P_K$ ), perhaps making them almost completely impermeable, we have an explanation of the falling off with time, and, moreover, a partial explanation of the discontinuity of permeability distribution amongst the cells. Thus, as soon as the cells are added to the solution of the potassium salt, all of them begin to take in potassium and the average increase per cell is high; however, those cells which have the lowest  $P_K$  and the highest permeability to sodium, ( $P_{Na}$ ), will shrink and therefore take in potassium at a progressively reduced rate so that the average intake per cell will decrease, i.e. the curve for penetration will become abnormally concave to the abscissae. If this explanation, which attributes to the escape of sodium the falling off of  $P_K$  with time, and consequently the division of the cells functionally into two parts, is correct, we may expect that an agent which inhibits this escape will prevent both of these manifestations. Narcotics, we have already said, reduce the  $P_K$  of the dog erythrocyte; they also affect  $P_{Na}$  in the same sense but to a much greater degree; thus in 0.6% *n*-butyl carbamate  $P_{Na}$  is reduced to about one-sixteenth of its value in isotonic potassium chloride alone, whilst  $P_K$  is only about halved. If we return to Fig. 4 we note that the curve for potassium penetration differs little from the theoretical one constructed from equation (1) and drawn in broken lines (the deviations from the theoretical curve strongly suggest that  $P_K$  is increased with the swelling of the cells, which becomes appreciable after a few hours). Furthermore, it was observed that no layer formation took place, and that when haemolysis

began the average potassium concentration per cell was close to that which would be expected were all the cells permeable to potassium and impermeable to sodium.

In further support of the suggestion that shrinking of the cells causes a reduction in the permeability to potassium, it was found that in 0.165 *M* potassium chloride, made hypertonic with glucose so that the osmotic pressure was equivalent to that of 0.22 *M* potassium chloride, the  $P_K$  decreased from a value of  $4.8 \times 10^{-18}$  to one of  $3.7 \times 10^{-18}$  g. mol./ $\mu^2$ /min./g. mol./l. concentration difference; if it is appreciated that the loss of half the sodium content of a cell causes it to shrink as much as a normal cell would when placed in 0.33 *M* salt solution, it becomes clear that the reduction of  $P_K$  due to loss of sodium can be very great indeed.

It is probable that this single factor, the reduction of  $P_K$  with shrinkage of the cell, is not sufficient to account for the comparatively sharp differentiation of the cells into apparently potassium permeable and potassium impermeable divisions; earlier studies on the cat erythrocyte showed that sodium permeability is markedly susceptible to changes in the degree of swelling of the cells, being completely inhibited in hypotonic solutions and markedly accelerated in hypertonic solutions; it was therefore thought that, by analogy, the swelling of the dog erythrocytes would cause them to become impermeable to sodium or would at least retard the loss. This was indeed found to be the case; thus in 0.16 *M* potassium chloride  $P_{Na}$  was  $1.49 \times 10^{-18}$ , whilst in 0.13 *M* solution it was reduced to  $0.87 \times 10^{-18}$ , i.e. to just over one-half its value; furthermore, in 0.22 *M* the rate was increased to  $2.00 \times 10^{-18}$ . These two factors together, the reduction of  $P_{Na}$  in the swollen cells and the reduction of  $P_K$  in the shrunken cells, together with an increase in  $P_{Na}$  in the shrunken cells, can be expected to give the phenomenon of partial haemolysis as it is observed in isotonic potassium chloride.

If this viewpoint is correct, we may expect that any factor which increases  $P_K$ , whilst at the same time leaving  $P_{Na}$  unchanged or decreased, should increase the limiting degree of haemolysis approached at infinite time, since this depends, as we have seen, on the ability of the potassium to enter rapidly enough to overcome the shrinking effect of sodium loss. In Fig. 1 we note that the limiting degree of haemolysis in the various salts is in the order  $CNS > NO_3 > Br > Cl > acetate$ , being about 80% in the case of thiocyanate and only 16% in the case of acetate. From Fig. 2 we see that the permeabilities to potassium are in the same order, and the order for sodium permeability in the same specimen of blood was as follows:

Acetate	>	Cl	>	Br	>	$NO_3$	>	CNS
3.0		1.7		1.4		1.1		—

where the figures represent the actual values of the permeability constants  $\times 10^{13}$ . Once again the deductions from theory are confirmed by experiment.

It has been pointed out earlier that the total amount of potassium in the cells, when haemolysis begins, varies under different conditions (Figs. 2-4). This apparent contradiction may now be shown to be a necessary consequence of the theory of haemolysis proposed here. The actual amount of potassium gained by all the cells, at the point when the first group of cells is ready to haemolyse, will obviously depend not only on the amount in this particular group but in the remaining ones too; if these are not shrunken below the limit at which penetration of potassium becomes negligible, it is clear that they will all have gained appreciable amounts during the period, and therefore the total amount of potassium gained by the suspension will be high; if, on the other hand, a large proportion has shrunk during the period up to the beginning of haemolysis the reverse will hold. Therefore as a rough generalization we may say that increasing  $P_K$  and decreasing  $P_{Na}$  should have the effect of increasing the average amount of potassium per cell at the beginning of haemolysis. This is confirmed by Figs. 2 and 4 and also by Fig. 3, since it has been observed that stored blood not only shows an increase of  $P_K$  but also a decrease in  $P_{Na}$ .

A further corollary of the theory is that the rate of haemolysis in potassium chloride solutions should increase with the dilution of the latter, since the amount of potassium which must enter a swollen cell to cause it to haemolyse is smaller than the amount required to haemolyse a normal or shrunken one. This factor will be partly compensated by the reduced concentration difference of potassium across the membrane in the more dilute solutions, but a simple calculation shows that the latter factor is relatively unimportant; thus if  $x$  is the concentration of the potassium chloride solution,  $y$ , the amount of potassium required to penetrate that volume of cells containing 1 l. of water, in order to cause haemolysis, is given by

$$y = (2x - 0.165);$$

the rate at which potassium enters the cells,  $dy/dt$ , varies as  $1/x$ , other things being equal. A simple substitution shows that if  $x$  is reduced from an isotonic value of 0.165 mol/l. to 0.100 mol,  $y$  is decreased from 0.165 to 0.035, i.e. nearly fivefold; on the other hand,  $dy/dt$  is only decreased by the ratio 0.100/0.165, i.e. not to one-half; hence we may expect the rate of haemolysis in 0.100 M KCl to be more than twice that in 0.165 M KCl. Similarly, it can be shown that in 0.22 M potassium chloride the rate of haemolysis should be reduced to about eight-tenths of its value in 0.165 M solution. In addition, the final degree of haemolysis

approached at infinite time should increase as  $x$  decreases, since the theory demands that with an increased proportion of swollen cells initially, fewer cells will have a chance of shrinking sufficiently to become impermeable to potassium.

The general correctness of these deductions is illustrated by the curves in Fig. 8, which show that in 0.10 *M* potassium chloride both the rate of haemolysis, and the final amount approached at infinite time, are greatly in excess of those in 0.165 *M* solution; similarly in hypertonic potassium chloride (0.22 *M*) the final degree of haemolysis is definitely

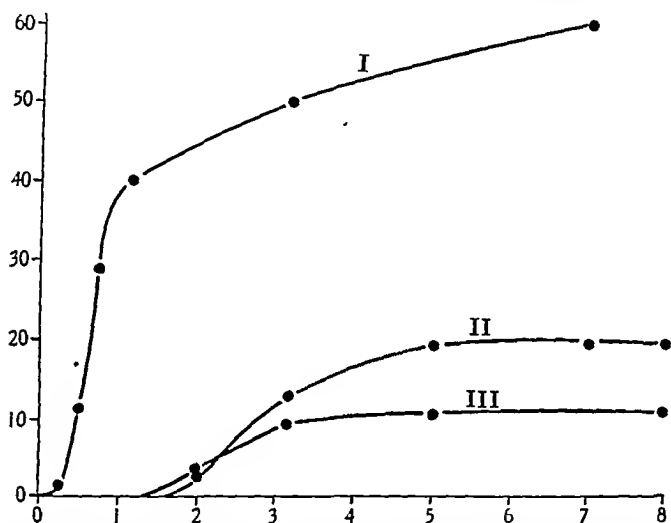


Fig. 8. Haemolysis of dog erythrocytes in 0.10 *M* (curve I), 0.165 *M* (curve II) and 0.22 *M* (curve III) potassium chloride solutions. Ordinates: percentage haemolysis. Abscissae: time in hours.

less than in isotonic solution, but in the early stages there is apparently a slight increase in rate. The above calculation ignores the effects of changed tonicity on sodium permeability and also any possible effect of a changed Na : K concentration ratio in the medium; consequently little more than a qualitative agreement between theory and experiment can be expected, and it is likely that the slightly increased rate of haemolysis in 0.22 *M* solution, as compared with 0.165 *M* solution, is due to an effect of the changed Na : K ratio.

It may now be asked whether the permeability of the dog erythrocyte to cations measured here is a normal characteristic of the cell, or whether it is not due to the disturbed Na : K ratio of the medium. To test this point, the gain of potassium and the loss of sodium of dog erythrocytes in 1 hr. have been measured in media containing proportions of sodium to potassium varying from less than 1:10 (the condition when 1.4 ml.



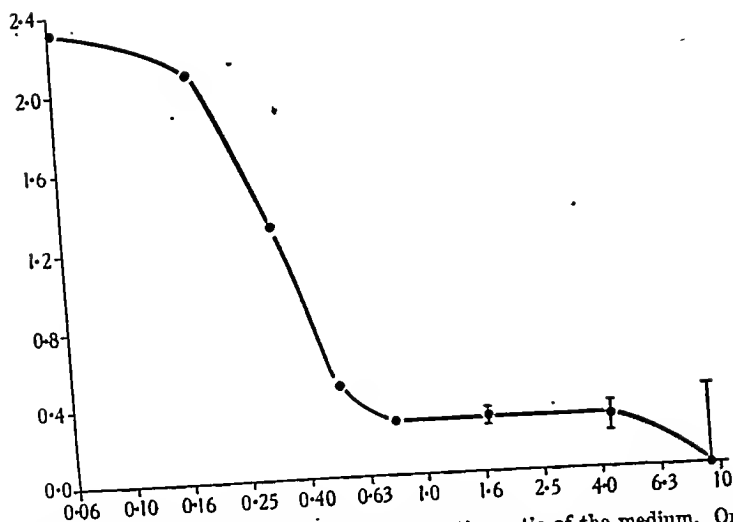


Fig. 9. Variation of  $P_K$  with the Na:K concentration ratio of the medium. Ordinates:  $P_K$  in g. mol./ $\mu^3$ /min./mol/l. concentration difference  $\times 10^{18}$ . Abscissae: Na:K concentration ratio. Note: abscissae are plotted on a logarithmic scale.

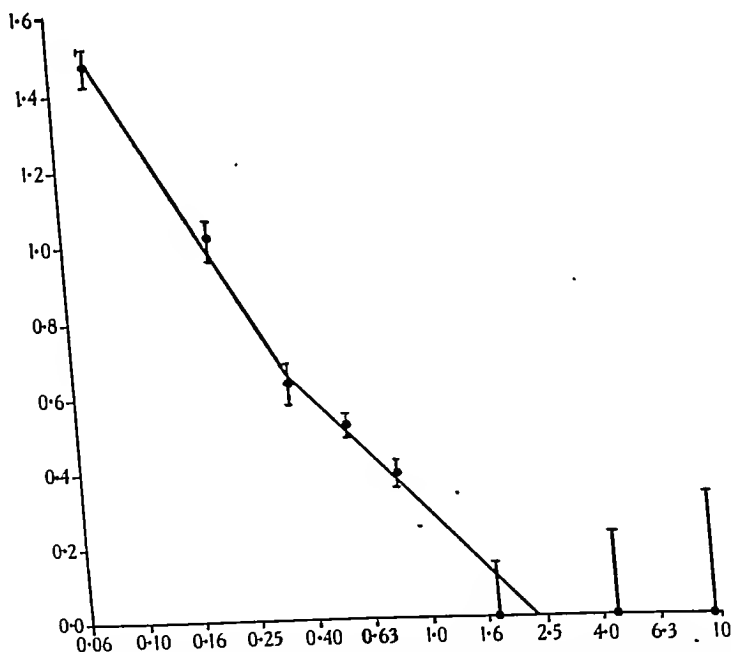


Fig. 10. Variation of  $P_{Na}$  with the Na:K concentration ratio of the medium. Ordinates:  $P_{Na}$  in g. mol./ $\mu^3$ /min./mol/l. concentration difference  $\times 10^{18}$ . Abscissae: Na:K concentration ratio. Note: abscissae are plotted on a logarithmic scale.

of whole blood are added to 10 ml. of isotonic potassium chloride) to nearly 10:1. The  $P_K$  and  $P_{Na}$  calculated from these values, assuming that the amount gained or lost in 1 hr. is a sufficiently accurate measure of  $dS/dt$ ,<sup>1</sup> have been plotted against the Na:K ratio in Figs. 9 and 10; for convenience, a logarithmic scale is used for the abscissae. The curves show unmistakably that both  $P_K$  and  $P_{Na}$  are dependent on this ratio and that they decrease to very low values as this approximates to that found in blood plasma (ca. 15:1); unfortunately, as the ratio is increased, the error in the determination of the permeability constant increases, owing to the small amounts of cation actually gained or lost under these conditions; the average errors are indicated in the figures (in the other figures of this paper the average error is amply covered by the sizes of the points of the curves). The results suggest that in a medium of physiological Na:K ratio the cells would be impermeable to sodium and possibly also to potassium. In this respect the dog erythrocyte differs markedly from that of the cat, since in this case  $P_K$  is approximately independent of the Na:K ratio of the medium.

#### DISCUSSION.

The results described in this paper strongly indicate that the haemolysis in potassium solutions is an osmotic, rather than a chemical, effect of the changed Na:K ratio of the medium. The fact that haemolysis does not proceed to completion, of itself suggests that the action of the potassium salt is different from that of the lysins properly so called, and is explained by the assumption that in any given potassium salt a certain proportion of the cells lose sodium more rapidly than they gain potassium and thereby shrink; this shrinkage of the cells favours the further escape of sodium and retards the penetration of potassium, so that after a time these cells lose practically all their sodium and take in potassium at a negligible rate. These cells are therefore those which do not haemolyse within the periods of observation (up to 8 hr.), and their proportion to the whole is determined mainly by the average relative values of  $P_K$  and  $P_{Na}$  of the cells in their initial states, and therefore should vary considerably from salt to salt according as the anions affect these two quantities. The order of effectiveness in causing haemolysis is, as we have seen, the order of the Hofmeister series, thiocyanate being most effective and acetate least. In the past [vide Höber, 1924] such an observation

<sup>1</sup>  $dS/dt$  should, of course, be calculated from the initial slope of the curve of potassium penetration or sodium loss; in this experiment such a procedure was not feasible; appreciable errors due to this approximation will only occur with the low Na:K ratios and will tend to make  $P_K$  and  $P_{Na}$  too low. Where the escape of sodium was very slow indeed a period of 3 hr. was actually used, as otherwise the losses of sodium would have been obscured by the experimental errors in their determination.

would have been interpreted as showing that the hydrating salts, such as thiocyanate, 'loosen' the membrane to allow the escape of haemoglobin; in the light of the present results, the series must be regarded as being determined by two influences on ionic permeability ( $\alpha$ ) on  $P_K$  and ( $\beta$ ) on  $P_{Na}$ . The reciprocal nature of the two effects is not easy to understand, but it is in conformity with earlier work on the cat erythrocyte [Davson, 1940b].

The cells, in which  $P_K$  is initially greater than  $P_{Na}$ , swell; this swelling retards the escape of sodium and possibly accelerates the rate of potassium intake; and hence the amount of sodium lost from the haemolysed cells will be small, those cells which haemolyse early losing considerably less than those haemolysing later. The amounts of potassium penetrating the cells required to cause haemolysis will therefore vary and will be greatest in those which haemolyse last; this undoubtedly is the reason why the corrected curve in dotted lines of Fig. 6 shows a rapid falling off during the later stages of haemolysis; the 'correction', which assumes that the cells haemolysed when they contained 0.11 mol/l. of cells, is evidently too small for the cells which haemolyse late.<sup>1</sup>

The demonstration that, in the case of the dog erythrocyte, the destructive effect of potassium chloride is in reality an osmotic phenomenon, secondary to an increased permeability to potassium, suggests that there is a limit to the possible effects of a disturbance of the salt balance of the medium, and that the more obvious changes produced in tissues and cells such as haemolysis, turgescence of muscles, impaired ciliary action, etc., are really due to much less gross changes in cell structure than are observed, namely, to a change in the permeability relationships of the cells to ions. This suggestion enables us to explain why it is that most species of erythrocyte do not haemolyse in isotonic potassium chloride. In the cat erythrocyte, in which the electrolyte distribution is nearly identical with that of the dog, the cells, when placed in isotonic potassium chloride, are likewise permeable to both potassium and sodium; however, in this case the average  $P_{Na}$  is considerably greater than  $P_K$  (often as much as five times), so that the cells shrink rather than swell and therefore no haemolysis takes place. With the ox erythrocyte, which likewise contains considerably more sodium than potassium, it is found that apart from an initial loss of sodium and gain of potassium [Davson, 1934] no continuous penetration of potassium or loss of sodium takes place in isotonic potassium chloride, and therefore this solution is

<sup>1</sup> A possible objection to the present theory of haemolysis is the absence of an appreciable slowing of the loss of sodium with time in the initial stages (Fig. 7). This could be explained, however, on the grounds that the slowing of escape due to a certain proportion of the cells becoming slowly impermeable to sodium (as a result of swelling) is compensated by the increase in permeability of the remainder due to their shrinking.

not haemolytic in respect to the ox erythrocyte. In the rabbit and human cells the concentration of potassium within the cells is approximately equal to that in isotonic potassium chloride solution, and we may therefore not expect penetration of potassium, as the necessary concentration gradient is lacking.

The action of a narcotic in postponing the haemolysis of the dog erythrocyte would in the past have been ascribed to a 'protective' action of the narcotic on the membrane which prevents its rupture by potassium chloride. The results of this paper show that the effect of the narcotic follows from its effect on  $P_K$ , and only in the sense that it inhibits the latter can it be said to exert any 'protective' action. This point may be strikingly illustrated by a comparison of the effects of 0.6% *n*-butyl carbamate on the cat and dog erythrocytes suspended in isotonic potassium chloride for a period of 5 hr. As we have seen, in the potassium chloride alone no haemolysis of the cat erythrocyte takes place, whereas, in the case of the dog erythrocyte, about 50% of the cells haemolyse. In the presence of the carbamate about 20% of the cat erythrocytes haemolyse whilst none of the dog erythrocytes do so. In the one case therefore, in ignorance of the permeability relationships of the cells, the narcotic would be said to be 'protective', whilst in the other it would be said to assist the potassium chloride in destroying the cell membrane. The explanation of these opposite actions of the narcotic more correctly resides in the fact that, in the case of the cat erythrocyte, narcotics completely inhibit sodium permeability but increase potassium permeability [Davson, 1940*a*], as opposed to the situation with the dog erythrocyte where the narcotic inhibits both sodium and potassium permeabilities.

In the present paper the main emphasis has been placed on the mechanism of haemolysis, for the reason that it is an instance of biological 'salt action' which lends itself to an interpretation in terms of the simpler and better understood concepts of ionic permeability, and should therefore demonstrate the necessity for an accurate knowledge of the ionic permeability relationships of any cell before any conclusions regarding the possible action of salts on it can be drawn. Instances of remarkable changes in permeability to sodium and potassium have been described, some of which are similar to those already described in the case of the cat erythrocyte whilst others differ markedly from these; however, a detailed comparison of the ionic permeability relationships in the two cells will be left to a later paper.

# SUMMARY

The haemolysis of the dog erythrocyte in isotonic solutions of potassium salts has been studied. The following points have been demonstrated:

1. The rate, and the final degree, of haemolysis vary with the anion of the potassium salt, being greatest in thiocyanate and least in acetate.

2. There is a parallelism between the magnitude of the permeability of the cells to potassium ( $P_K$ ) and the rate of haemolysis under a variety of conditions.

3. The amount of sodium which escapes from the cells in isotonic potassium chloride is about equal to the amount of potassium which penetrates during the period up to the beginning of haemolysis.

4. In isotonic solutions of potassium salts the cells separate themselves functionally into two groups: (a) one which gains potassium rapidly and loses sodium slowly, and (b) one which loses sodium rapidly and gains potassium slowly. Group (a) consists of the cells which haemolyse whilst those in group (b) do not. This separation is determined by three main factors: (i) the relative values of  $P_K$  and  $P_{Na}$  vary among the cells, so that there is a group in which  $P_K$  is greater than  $P_{Na}$  and one in which  $P_{Na}$  is greater than  $P_K$ ; (ii) the swelling of the cells progressively retards the escape of sodium; (iii) the shrinking of the cells progressively retards the penetration of potassium.

5. The relative sizes of groups (a) and (b) under any conditions depend on the average initial values of  $P_K$  and  $P_{Na}$ ; if  $P_K$  is initially very much greater than  $P_{Na}$  the proportion of (a) is large and the final degree of haemolysis is high.

6.  $P_K$  and  $P_{Na}$  depend on the Na:K concentration ratio of the medium, being high when the ratio is small and vice versa.

It is concluded that the haemolysis of the dog erythrocyte in isotonic solutions of potassium salts is an osmotic phenomenon due to the penetration of potassium.

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PREPARATION AND SOME PROPERTIES  
OF HYPERTENSIN (ANGIOTONIN)BY P. EDMAN, U. S. VON EULER, E. JORPES AND  
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The blood-pressure raising effect of fresh kidney extract was discovered by Tigerstedt & Bergman [1898], who ascribed this action to a heat-unstable undialysable protein substance which they named renin. Later investigations [Kohlstaedt, Helmer & Page, 1938] showed that this substance required the presence of some other substance occurring in the blood for the development of its vasoconstrictor action. When this 'activator' and renin were allowed to interact a pressor substance was formed, which was heat stable and alcohol soluble. The new active substance was called angiotonin [Page & Helmer, 1939, 1940]. Braun-Menendez, Fasciolo, Leloir & Muñoz [1939] in Houssay's laboratory found that venous blood from the ischaemic kidney contained a heat-stable, dialysable pressor substance which they named hypertensin, and showed also that incubation of a certain fraction of blood plasma or serum with renin led to the formation of a substance with similar properties [1940a]. It is now generally accepted that renin acts as an enzyme on a substrate in blood belonging to the pseudoglobulins, forming a heat-stable, dialysable vasoconstrictor substance (hypertensin, angiotonin).

As to the chemical nature of this substance, Page & Helmer [1940], as well as Braun-Menendez *et al.* [1940b], have made certain suggestions. The former authors [1940] claimed that they had isolated a crystalline picrate and oxalate of the active agent supposed to be a base. Page, Helmer & Kohlstaedt [1940] had previously shown that the pressor agent was not identical with tyramine. Braun-Menendez *et al.* only stated that the active substance behaves like a polypeptide, being dialysable, precipitable with ammonium sulphate and destroyed by proteolytic enzymes like pepsin and trypsin.

In the present paper a report is given of some experiments undertaken in order to study the formation and some properties of this substance.

## PREPARATION OF HYPERTENSIN

The crude hypertensin (angiotonin) was prepared as described by Page & Helmer [1940].

The renin was prepared from fresh hog-kidney cortex. The material was ground, extracted with 2 vol. of acetone and left to dry at room temperature. After drying, the kidney substance was finely powdered and extracted twice with 5 and 3 vol. respectively of a 2% sodium chloride solution. To the extract, glacial acetic acid was added to 2%, the precipitate filtered off and the clear filtrate dialysed against running water for 24 hr. The salt-free renin solution was then concentrated in vacuo to 1/10 of its volume and filtered again if necessary. 500 g. dry powder gave about 250 ml. renin solution. When tested on the atropinized cat under chloralose anaesthesia, 0.25 ml. of this solution produced a rise in blood pressure of some 50 mm. Hg. Since this crude solution showed no untoward side reactions it was used for the preparation of hypertensin without further purification.

The globulins were obtained most easily from defibrinated horse blood, where the rapid sedimentation rate made centrifuging unnecessary. An equal volume of a saturated ammonium sulphate solution was added to the serum and the precipitate collected on filter paper. It was placed in cellophane sacs and dialysed under toluene at room temperature against running tap water for 48-60 hr. The temperature of the tap water was fairly low even in the summer and kept the system cool. The contents of the cellophane sacs were emptied and mixed twice during the dialysis, and the sacs were kept in movement mechanically the whole time. The volume of the final globulin solution after dialysis was somewhat smaller than that of the serum. No adjustment of the pH of the globulin solution seemed to be necessary before adding the renin.

15-20 l. globulin solution obtained from 20 to 25 l. horse serum were heated in an enamelled jar to 40° C. and 750 ml. to 1 l. renin solution (from 1.5 to 2 kg. dried pig-kidney cortex) were added. After 10 min. the globulin-renin solution was quickly denatured by heating in a steam bath. The temperature of the solution rose to 70° C. within 5-6 min. The heating was continued for 15 min. After cooling, the globulins were filtered off and the precipitate washed with water. The filtrate was concentrated in vacuo to 500 ml. and 4 vol. of alcohol were added. The precipitate was removed and the filtrate concentrated to 50 ml. The solution was made faintly alkaline to litmus with 1N NaOH, and 500 ml. of dry MeOH were added. After storage in the ice-box for 12-18 hr. the precipitate was removed. This precipitate was inactive. Then 2 l. dry peroxide-free ether were added. The precipitate was collected in centrifuge tubes, washed with ether and dried in vacuo. The yield per litre horse serum was 100 mg. in one preparation and 200 and 250 mg. in two following batches.

All of them showed the same pressor activity, equivalent to 0.7-0.8 mg. tyramine phosphate (T.P.) per mg. dry substance.

A second precipitate of the same order of magnitude or somewhat smaller could be obtained on adding more ether to the filtrate. It showed a 7-8 times weaker activity, 1 mg. corresponding to 0.1 mg. T.P.

Somewhat stronger preparations were obtained if the concentrate, after the removal of impurities with 4 vol. of alcohol, was fractionated with ammonium sulphate. To the concentrate, 400 ml., obtained from 10 l. of globulin solution ammonium sulphate was added to half and full saturation. The precipitates were collected, sucked dry and each was dissolved in 50 ml. of water. Dry methyl alcohol was added to 90 % and the precipitate removed. To the mother liquor 3 vol. of peroxide-free ether were added. The precipitates

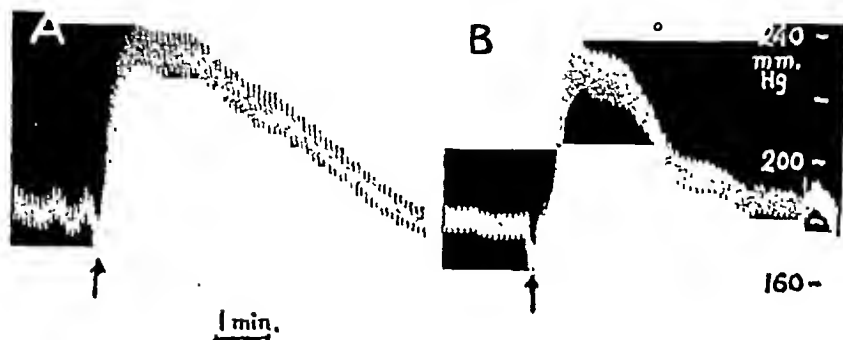


Fig. 1. Cat, chloralose, 1 mg. atropine sulphate per kg. intravenously. Blood pressure from the femoral artery. A, 0.25 mg. tyramine phosphate; B, 0.2 ml. hypertensin solution.

removed with methyl alcohol weighed 3.6 and 0.9 g. respectively, the former being inactive. The active fractions precipitated with ether weighed 500 and 270 mg. and showed a pressor activity corresponding to 1 and 2 mg. T.P. respectively per mg. dry substance.

All preparations were tested on cats under chloralose anaesthesia after the intravenous injection of 1 mg. atropine per kg. body weight. In order to obtain a quantitative measure of the effects tyramine was used as a reference substance. Though there was a certain difference in the type of action (Fig. 1) the relative activities of tyramine and hypertensin could easily be compared.

#### THE PURIFICATION AND PROPERTIES OF HYPERTENSIN

As to the chemical nature of the pressor substance, the findings of Braun-Menendez *et al.* that the hypertensin is destroyed through the influence of pepsin and other proteolytic enzymes were confirmed. It seems very probable, therefore, that the hypertensin is of protein nature.



To a 1 % solution of pepsin (Parke, Davis and Co.) 1 : 3000 in  $N/100$  HCl an equal volume of a hypertensin solution was added. The activity was completely destroyed in 2 hr. at 37° C. and in 6 hr. at room temperature. In both cases; no loss of activity was experienced in using boiled pepsin and trypsin solutions under similar conditions. Nor was there any inactivation of hypertensin if it was stored at room temperature in  $N/2$  hydrochloric acid or in  $N/2$  sodium hydroxide for 10 min. or, in ten times weaker solutions, for 1 hr.

Hypertensin is also precipitated by ammonium sulphate. Attempts at fractionating with this salt have so far been less successful because the activity was not to be found exclusively in any definite fraction. The main part of the hypertensin was usually precipitated first at full saturation with ammonium sulphate, but a certain percentage of the activity came down at 40 % saturation.

#### THE CATAPHORETIC MOBILITY OF HYPERTENSIN

The protein structure thus being the most probable one, the isoelectric properties of hypertensin were studied in cataphoresis experiments with the Tiselius apparatus. For this purpose a sample of hypertensin was used 1 mg. of which gave the same pressor effect as 2 mg. of T.P.

Four observations were made between  $pH$  6 and 7. The hypertensin behaved like an amphoteric electrolyte, and its isoelectric point was between  $pH$  6.3 and 6.5. The electrophoretic mobility of the hypertensin could not be determined accurately because of its high diffusibility even outside the electric field. The electrophoretic behaviour of hypertensin thus supports the opinion that hypertensin is of protein nature.

These experiments, however, do not allow any definite conclusions about the true isoelectric properties of the hypertensin itself, because it is a common observation that contaminating proteins may influence the cataphoretic mobility and may give misleading results.

The further purification of the hypertensin preparations was not easy, mainly because of its instability. The activity was very often lost during the manipulations. Thus, drying after previous treatment with acetone or ether resulted in considerable loss. The active substance was adsorbed on permutit only to about 50 % or less. It passed through the 'Cella feinst' filter, but the filtration under pressure proceeded very slowly.

Repeated attempts were made to use electrodialysis through a parchment membrane. In single small runs 80 % of the active substance was found in the cathode fluid. In others only 45 %. Acetone, cooled with solid carbon dioxide, was passed through the cooling spiral. In most of the experiments, however, the activity was lost and the process could not be applied on a larger scale. In one experiment the pressor effect of the substance obtained on drying the

neutralized cathode fluid was fairly high. Per mg. organic substance it corresponded to 5 mg. tyramine phosphate.

The purest preparations gave no reaction for phenol with ferric chloride and no discoloration in alkaline solution. The carbazole reaction of Dische was positive, indicating the presence of a certain amount of carbohydrate.

#### SUMMARY

The claims of Braun-Menendez *et al.* that the blood-pressure effect caused by renin is due to a proteose split off from the serum globulins through the action of renin have been confirmed. The active substance, hypertensin or angiotonin, is precipitated with ammonium sulphate, destroyed by pepsin and pancreatic extracts, and is of amphoteric nature with an isoelectric field between pH 6.3 and 6.5. This indicates that it is a proteose. Its formation through an enzymic action on the biologically inactive serum globulins may throw some light upon the formation of secretin, cholecystokinin, substance P of Euler and Gaddum, and similar active substances of protein nature.

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# AN ACTION OF ADRENALINE ON TRANSMISSION IN SYMPATHETIC GANGLIA, WHICH MAY PLAY A PART IN SHOCK

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We have had two reasons for approaching this problem. One of us [Burn, 1932] showed that when the hindleg of a dog was perfused with defibrinated blood through a cannula tied in the abdominal aorta 3-4 cm. above the bifurcation, the vasoconstriction produced by stimulating the lumbar sympathetic chain was very small. When adrenaline was added to the perfusing blood, however, the effect of the same stimulation was greater, and this augmentation outlasted the effect of the added adrenaline on the vascular tone. The observations were taken to mean that sympathetic stimulation was effected by the liberation of adrenaline from the post-ganglionic endings, and that in the preparation used the necessary store of this adrenaline was depleted, until it was restored by addition of adrenaline to the blood. Thus, the augmentation of the effect of sympathetic stimulation was thought to be due to a change at the post-ganglionic terminations.

Recently, Dr P. Glees called our attention to a paper by Stöhr [1939] in which large-scale drawings of sympathetic ganglia showed the ganglion cells surrounded by what Stöhr described as chromaffine tissue. Dr Glees himself obtained photographs showing similar small cells. Reflexion suggested that if chromaffine cells were present in sympathetic ganglia, they presumably exerted some function. What function could adrenaline have in sympathetic ganglia? The question recalled the observations which have just been described, in which the stimulation was applied to the preganglionic fibres of the sympathetic chain. It was clear that the augmentation of the effect of stimulation produced by adrenaline might have been due to a change in the ganglion whereby transmission was improved.

Our second reason for studying this question was that we have recently [Bülbring & Burn, 1941] made observations on the transmission of impulses in the spinal cord, and have observed three effects of adrenaline. We found that adrenaline facilitated the action of small doses of acetylcholine in causing

a discharge of motor impulses from the spinal cord. We found that adrenaline, by an action on the spinal cord, augmented the size of the flexor reflex. Thirdly, we found that adrenaline modified the action of prostigmine on the flexor reflex; in the presence of adrenaline, prostigmine powerfully augmented the flexor reflex; in its absence prostigmine was without effect.

Thinking it likely from the evidence we obtained that synaptic transmission in the spinal cord is, in part at least, by acetylcholine, and that these effects of adrenaline were modifications of such a transmission, we decided to examine other parts of the body, where impulses are transmitted by acetylcholine, for similar effects of adrenaline. We first studied the transmission at the neuromuscular junction in skeletal muscle, and looked to see whether adrenaline would modify the action of prostigmine. We found [Bülbring & Burn, 1942] that it did. When maximal single shocks are applied to the sciatic nerve, and the tension in the gastrocnemius is recorded, if the rate of stimulation is slow, the injection of prostigmine leads to an increase in the tension. We found that when adrenaline was injected this increase was much greater. On the other hand, when more rapid stimulation was used the increase in tension caused by prostigmine disappeared when adrenaline was injected. Thus, at the neuromuscular junction adrenaline augmented or diminished the action of prostigmine according to the rate of stimulation.

The second site at which transmission is effected by acetylcholine is the sympathetic ganglia. What effect had adrenaline on transmission here? Marrazzi [1939 *a, b*] has published evidence that adrenaline causes depression and inhibition of ganglionic transmission. He recorded the action potentials in the post-ganglionic fibres coming from the superior cervical ganglion, and observed that when adrenaline was injected these action potentials were reduced. This reduction was observed when the dose of adrenaline was as small as  $5\mu\text{g.}$ , and when adrenaline was not injected but liberated in the body by stimulation of the splanchnic nerve. Marrazzi showed that occlusion of the circulation to the ganglion for 2-3 min. produced no depression and, therefore, concluded that the adrenaline effect could not be due to diminution of the blood supply to the ganglion.

## RESULTS

*Perfusion experiments.* We have made observations by three different methods, all of which have given similar results. Our main evidence has been obtained with a preparation we have already described in detail [Bülbring & Burn, 1941]. This consists of a perfusion scheme in which there are two circulations of defibrinated blood entirely separate from one another. For the purpose of these experiments the essential feature of the one circulation is that it supplies the sympathetic ganglia, while the other supplies the vessels in which the post-ganglionic fibres terminate. We were thus able to study the

effect of adrenaline on the transmission of impulses through the ganglia, while excluding any change at the post-ganglionic terminations.

The actual observations were made in the lower half of the body of a dog from which the viscera were removed. The one circulation passed through a stretch of aorta from the bifurcation to just above the renal arteries (which were tied), and supplied the sympathetic chain through the spinal arteries; the second circulation supplied the vessels of the left hindleg. All vascular connexions between the two circulations were tied, while the nervous connexions remained untouched. The arterial pressures were recorded with a mercury manometer from each arterial cannula. Changes in the arterial pressure in the ganglion circuit were modified by an artificial resistance, tied in the upper end of the aorta, acting as a shunt to prevent any excessive rise of pressure in a relatively small circulation. By means of this shunt it was possible to alter the adrenaline content of the blood without greatly changing the perfusion pressure and consequently the blood supply to the ganglia.

*Effect of adrenaline.* The course of an experiment may be followed in Fig. 1. The upper record shows the changes in the venous outflow from the leg vessels, while the middle record shows the corresponding changes in arterial resistance in these vessels. The lower record shows the arterial resistance in the circuit supplying the ganglia. The first four sections (*a, b, c, d*) show the effect of a steady addition of adrenaline at the rate of 0.005 mg., later 0.003 mg./min. to the reservoir supplying blood for the leg. At the points marked by the signal the sympathetic chain was stimulated at the level of the kidney from an induction coil for periods of 10 sec., 32 break shocks being applied per sec. The vasoconstrictor effect of this stimulation in the hindleg is seen to increase with the rise in vascular tone produced by the adrenaline. During this stage of the experiment no adrenaline was added to the blood passing through the sympathetic ganglia, although a concentration of 0.05 mg./l. had been added before the perfusion began, and in the 40 min. elapsing between Fig. 1(*a*) and (*e*) was slowly disappearing. The effect of this disappearance first showed itself in Fig. 1(*e*) where, although the tone in the leg vessels was still higher than in (*d*), the effect of sympathetic stimulation was much reduced. Between (*e*) and (*f*) over a period of 30 min., adrenaline 0.003 mg./min. was added to the reservoir supplying blood to the ganglia. Stimulation of the sympathetic chain then produced a greatly augmented effect in the leg (*f*). The addition of adrenaline to the blood circulating through the ganglia was then stopped, while that to the blood for the hindleg was maintained, and 15 min. later in (*g*), the smaller response to stimulation was recorded once more.

In Fig. 2 the same influence of a varying concentration of adrenaline in the blood supplying the ganglia is shown. Throughout the period covered by the figure adrenaline was added to the blood for the hindleg at a constant rate (0.006 mg./min. to a volume of 600 c.c.). In (*a*) adrenaline was added at the

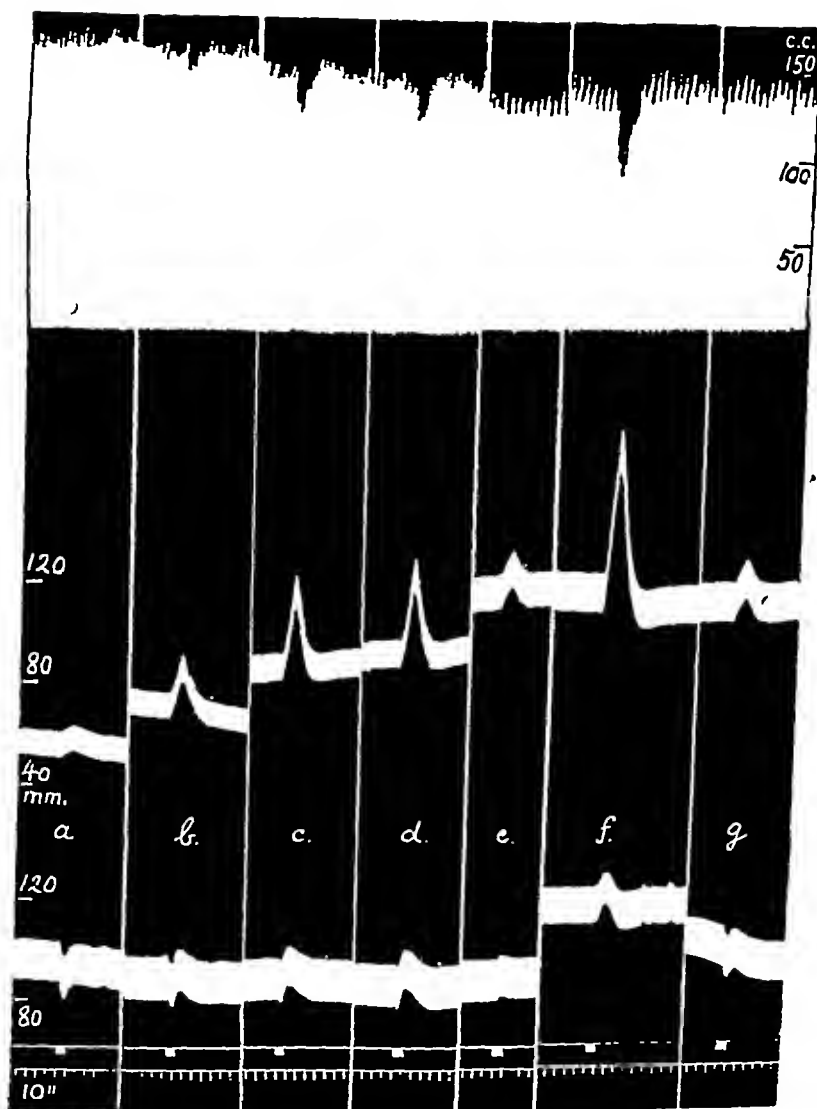


Fig. 1. Dog, double perfusion. Records from above downwards: Venous outflow in leg circuit; arterial resistance in leg circuit; arterial resistance in ganglion circuit. At signals stimulation of sympathetic chain, coil 15, 10 sec. The vasoconstrictor effects in the leg are shown, (a)-(e), when adrenaline was added to the leg circuit while it disappeared from the ganglion circuit. The response was augmented when adrenaline was added to the ganglion circuit (f), and became small again (g) when the adrenaline was stopped.

same rate to the blood for the ganglia, but between (a) and (b) this addition was stopped. During (b), (c) and (d) we recorded a diminishing response to

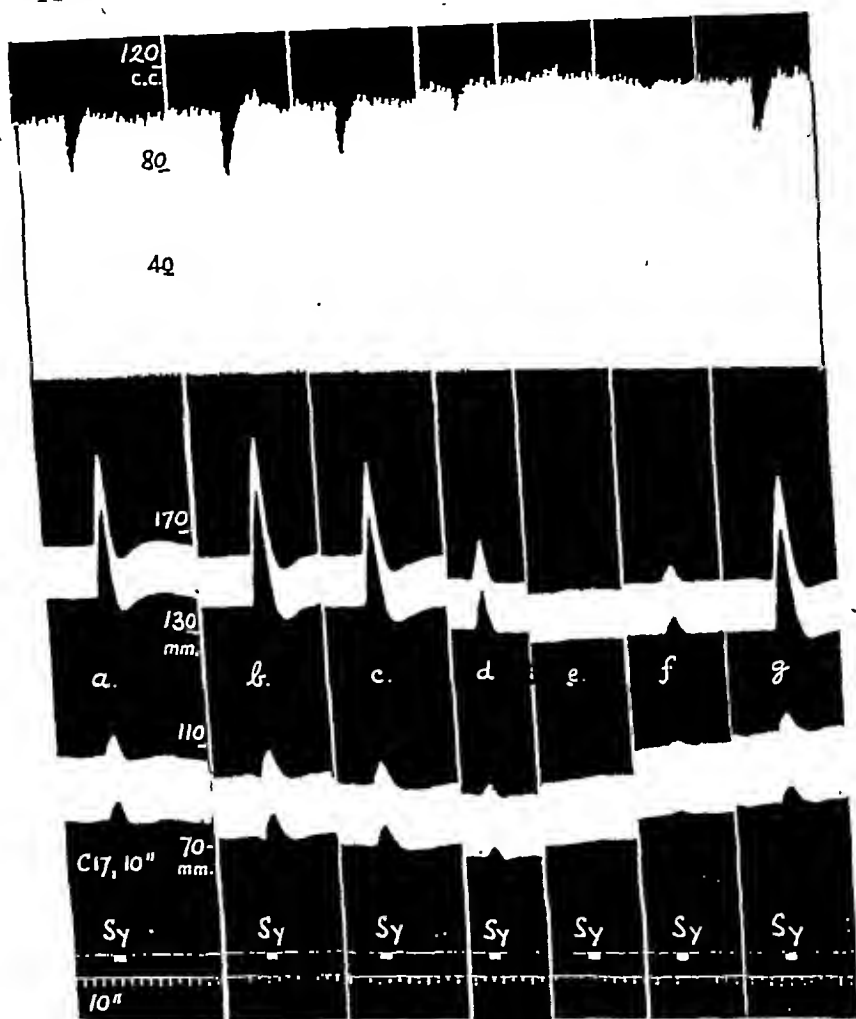


Fig. 2. Dog, double perfusion. Records as in Fig. 1. The vasoconstriction in the leg produced by sympathetic stimulation, coil 17, 10 sec., became less and disappeared when adrenaline disappeared from the ganglion circuit, (a)-(d), although maintained throughout at 0.008 mg./min. in the leg circuit. On adding adrenaline to the ganglion circuit, (e)-(g), the vasoconstrictor response in the leg reappeared.

sympathetic stimulation, which in (e) disappeared altogether. Adrenaline was then added to the blood for the ganglia once more, and, as shown in (f) and (g), the response to the sympathetic stimulation returned to its original height. The time elapsing between (a) and (g) was 1 hr.

*Effect of larger amounts of adrenaline.* The foregoing results indicated that adrenaline improved the transmission of impulses through the sympathetic ganglia. We obtained evidence, however, that when the amount of adrenaline was large, the opposite effect was obtained. This depression of transmission is illustrated in Fig. 3. Throughout this experiment the adrenaline addition to the leg circuit was kept constant at a rate of 0.005 mg./min., and the perfusion pressure remained steady. To the blood supplying the ganglia during the period from which (a) is taken, adrenaline was added at a rate of 0.003 mg./min. This rate was increased to 0.01 mg./min. between (a) and (b) and kept there for 30 min. from (b) to (d), after which it was stopped. The pressor response in the leg to stimulation of the sympathetic chain in (a) was 40 mm., but with the trebled adrenaline concentration in the blood to the ganglia, the response in (b), (c) and (d) fell to 30, 20 and finally 8 mm. As the concentration of adrenaline declined in (e), (f) and (g), the response increased again to 24, 35 and 64 mm.

*Effect of adrenaline on the stimulating action of acetylcholine on sympathetic ganglia and the suprarenal medulla.* We have carried out experiments to see if the above observations could be confirmed in the whole animal, and have used two different methods. It is well known that in the fully atropinized animal, the injection of large doses of acetylcholine causes a rise of blood pressure, which is due to stimulation of the sympathetic ganglia and the suprarenal medulla. In atropinized spinal cats the pressor effect of a given dose of acetylcholine, injected intravenously at regular intervals of 10–20 min., was found to be approximately constant. When a small dose of adrenaline was interposed between two injections of acetylcholine, the pressor effect of the acetylcholine was increased. Moreover, when successive doses of acetylcholine were given at shorter intervals of 3–5 min., their pressor effect steadily increased, and it appeared likely that this was due to the action of adrenaline liberated by one injection on the next. In Fig. 4A the increased pressor effect of 0.4 mg. acetylcholine is seen after 0.005 mg. adrenaline, the increase persisting for more than 20 min. The effect in this experiment was unusually well-marked, in most cats it was smaller, and in one it was not seen at all. The increase of the pressor effect of acetylcholine was only seen in animals with the suprarenal glands intact. It appeared immediately after the pressor effect of a small dose of adrenaline (0.0025–0.005 mg.) had passed off and persisted for 20 min. After slightly larger doses (0.01 mg.) there was at first a period during which the pressor effect of acetylcholine was decreased and the augmentation appeared only after 20 min.

The depression of the ganglionic effect of acetylcholine by large doses of adrenaline was observed with regularity both in animals in which the suprarenal glands were intact and in which they were removed. Fig. 4B, taken from the same experiment as Fig. 4A, illustrates this effect, when 0.03 mg. adrena-



line depressed the action of 0.6 mg. acetylcholine for 40 min. Still larger doses of adrenaline, especially when given by slow infusion, almost abolished the action of acetylcholine, injected after the infusion was stopped and when the pressor effect of the adrenaline had disappeared.

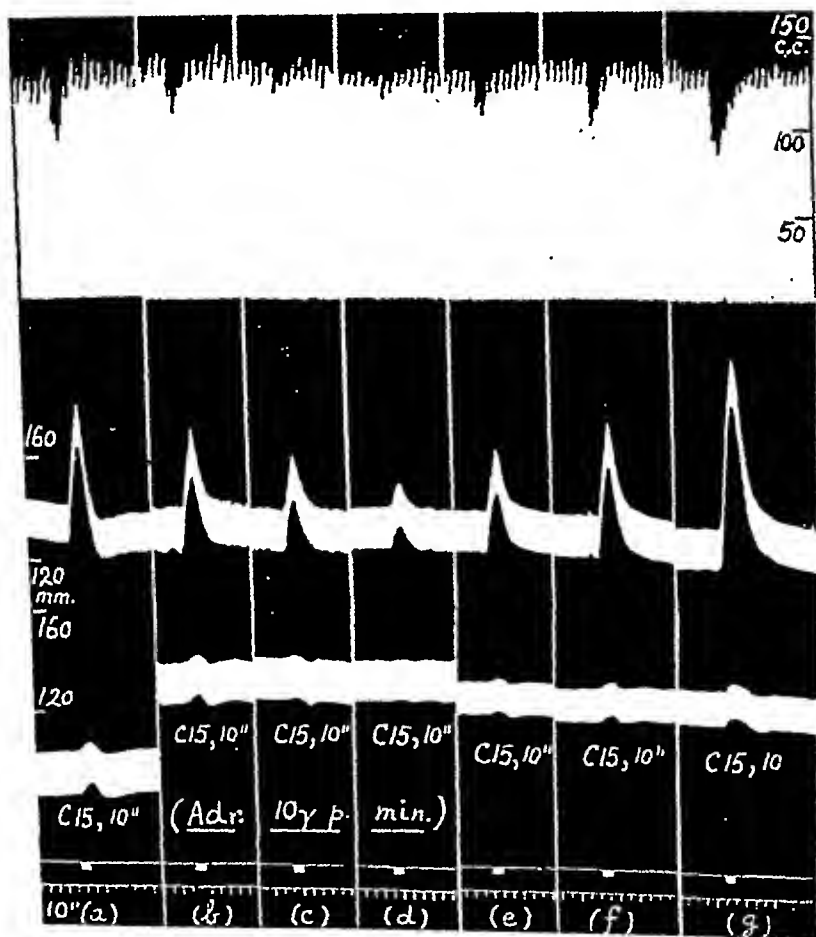


Fig. 3. Dog, double perfusion. Records as in Fig. 1. The vasoconstriction in the leg due to stimulation of the sympathetic chain is shown in (a) when adrenaline was infused, 0.005 mg./min. into the leg circuit and 0.003 mg./min. into the ganglion circuit. When the amount of adrenaline in the ganglion circuit was excessive, 0.01 mg./min., (b)–(d) a gradual diminution of the sympathetic effects was observed, which increased again (e)–(g) as the adrenaline tone was allowed to pass off.

During a slow infusion of adrenaline the pressor effect of acetylcholine was always either depressed or abolished. In the experiment illustrated in Fig. 5, adrenaline was infused into a spinal cat in which the suprarenals were excluded; the rate of infusion was 0.004 mg./min. and the blood pressure was maintained

at 120 mm. The injection of 2 mg. acetylcholine produced no appreciable rise, and indeed there was a slight fall despite the injection of 2 mg. atropine just

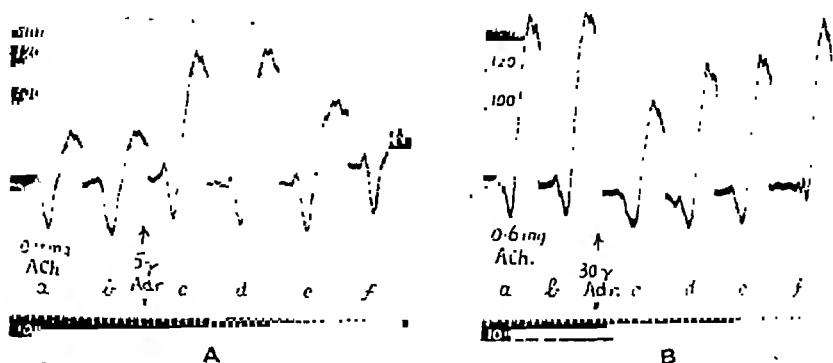


Fig. 4. Spinal cat, atropine. (A) Pressor effects of 0.4 mg. acetylcholine injected at 20 min. intervals. Just before (c) 0.005 mg. adrenaline was injected and augmented the effect of the acetylcholine. (B) Same experiment as (A), pressor effects of 0.6 mg. acetylcholine injected at 10 min. intervals. Between (b) and (c) 0.03 mg. adrenaline was injected and depressed the effect of the acetylcholine.

before. The infusion of adrenaline was then stopped, and after an interval the blood pressure was raised to the same level by infusing pituitary (posterior lobe) extract at the rate of 0.2 unit/min. The injection of 2 mg. acetylcholine then caused a sharp rise of blood pressure of 76 mm., showing that in the presence of pituitary extract the ganglionic stimulation was vigorous.

*Effect of adrenaline on the pressor effect of splanchnic stimulation.* The third method of studying the action of adrenaline was to stimulate the preganglionic splanchnic fibres in spinal cats in which the suprarenal glands were excluded. The splanchnic nerves were cut where they left the sympathetic chain in the thorax, and dissected up to the semilunar ganglia. The right splanchnic nerve was brought to the left side underneath the aorta, so that both splanchnics could be stimulated side by side. The mid-line incision through which the operation had been done was then sewn up to prevent exposure of the intestines, and a fresh lateral incision just above the left kidney was made to allow access to the splanchnics. These were laid on a shielded silver plate 6 mm. wide acting as a unipolar electrode, the second

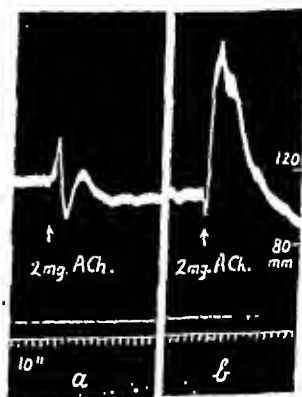


Fig. 5. Spinal cat, suprarenals excluded, atropine. The effect of injecting 2 mg. acetylcholine is shown in (a) during an infusion of adrenaline, 0.004 mg./min.; and in (b) during an infusion of pituitary posterior lobe extract, 0.2 unit/min.

electrode being placed under the skin nearby. The intestines were protected from exposure at this incision by careful packing with cotton wool. The nerves were stimulated at regular intervals, for 10–15 sec., by condenser discharges at rates which varied in different experiments from 8 to 48 per sec., and care was taken to see that the stimulation was maximal.

Unlike the pressor effect of acetylcholine which was augmented by adrenaline only when the vascular effect of the adrenaline had disappeared, the pressor effect of splanchnic stimulation was readily augmented during a continuous adrenaline infusion. Thus, in the presence of adrenaline, the same stimulus applied to the preganglionic fibres produced a much larger effect than before,

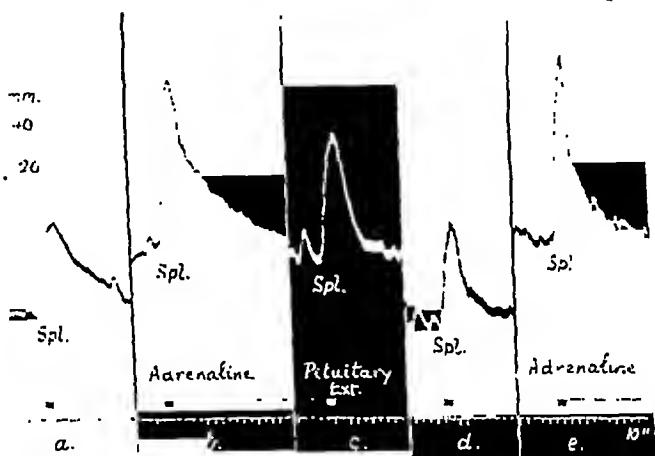


Fig. 6. Spinal cat; suprarenals excluded. The effect of stimulating both splanchnic nerves (48 per sec., for 10 sec.) is seen in (a) before; in (b) during an infusion of adrenaline 0.0025 mg./min.; in (c) during an infusion of pituitary posterior lobe extract 0.12 unit/min.; in (d) 37 min. later when the pituitary tone had disappeared; and in (e) once more during an infusion of adrenaline 0.0045 mg./min.

or than that produced in the presence of pituitary extract at the same height of blood pressure. In Fig. 6 adrenaline was infused at about 0.0025 mg./min. into the femoral vein and the effect of splanchnic stimulation was almost doubled, as shown by comparing the rise of pressure in (a) with that in (b). The adrenaline infusion was stopped, and an infusion of pituitary extract at a rate of 0.12 unit/min. was begun instead. When the blood pressure was about the same height as in (b), stimulation of the splanchnic nerves produced a rise in pressure (c) only a little greater than that in (a). The pituitary infusion was stopped, and when the blood pressure declined to the level in (a), the stimulation in (d) caused the same rise as in (a). Adrenaline was infused again at a higher rate of 0.0045 mg./min., and repetition of the stimulation (e) once more was followed by a much larger rise.

In view of the results with acetylcholine we were expecting that when the rate of adrenaline infusion was raised above 0.005 mg./min. we would find that the response to splanchnic stimulation would be depressed. This, however, did not happen, and indeed in the experiment from which Fig. 6 is taken there was no diminution of the response during 40 min. infusion at a rate of 0.015 mg./min. The response remained increased about 90–100 min. in height as in Fig. 6 (e). We found, however, that whenever the adrenaline infusion was stopped, and the blood pressure fell, the response to splanchnic stimulation became very small, e.g. 20 mm.

While an adrenaline infusion regularly increased the response to splanchnic stimulation, it scarcely altered the effect of a given dose of adrenaline. In

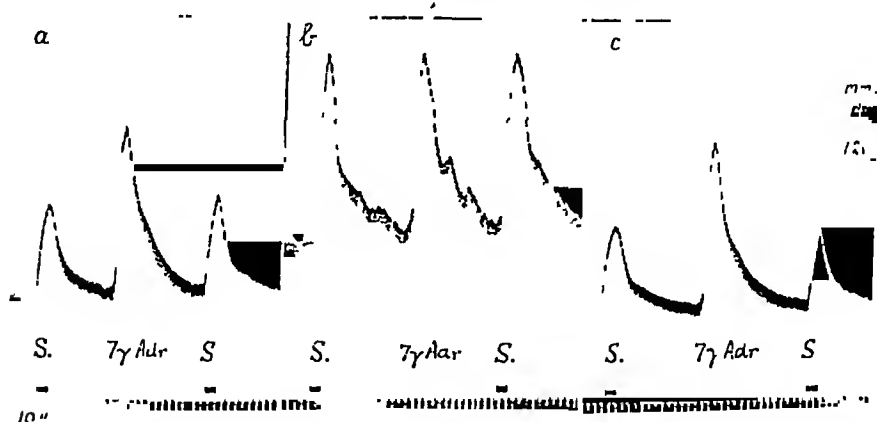


Fig. 7. Spinal cat, suprarenals excluded. *S*=stimulation of both splanchnic nerves (16 per sec for 15 sec.). The pressor effect of 0.007 mg. adrenaline is compared with that of splanchnic stimulation (a) before, (c) after, and (b) during an infusion of 0.0025 mg. adrenaline/min.

Fig. 7 (a) the pressor effect due to splanchnic stimulation was about half the size of that produced by 0.007 mg. adrenaline; in (b), while adrenaline 0.0025 mg./min. was infused, the splanchnic effect was more than doubled whereas the adrenaline effect was increased by only 10 %. When the adrenaline infusion was stopped (c) the splanchnic effect became less than its initial size, the adrenaline effect was still increased. In another experiment, the pressor effect of splanchnic stimulation, which was the same as that of 0.002 mg. adrenaline, was augmented during an adrenaline tone to the size of that produced by 0.01 mg. adrenaline. Increases of the pressor effect of adrenaline by 10–20 % have been observed during an adrenaline infusion; these were, however, always much smaller than the increases of the pressor response to splanchnic stimulation which ranged from 100 to 500 %.

To study the depressant action of larger amounts of adrenaline, we did not infuse it, but injected single doses, comparing the effect of stimulation when

the pressor action of the dose had passed off with that before it was given. The depression was regularly observed after 0.04 mg. or more. Whatever the size of the dose, the response was usually smallest 10–15 min. after the adrenaline pressor effect had disappeared, and it returned to its original height

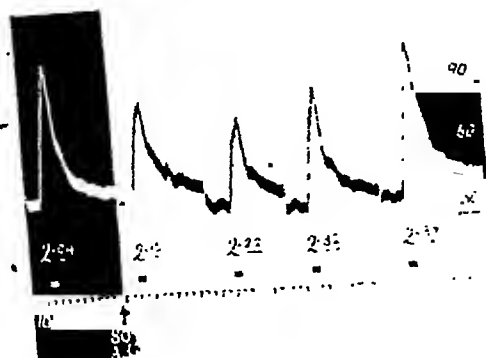


Fig. 8. Spinal cat, suprarenals excluded. Pressor effects of stimulating both splanchnic nerves are shown before and after the injection of 0.08 mg. adrenaline.

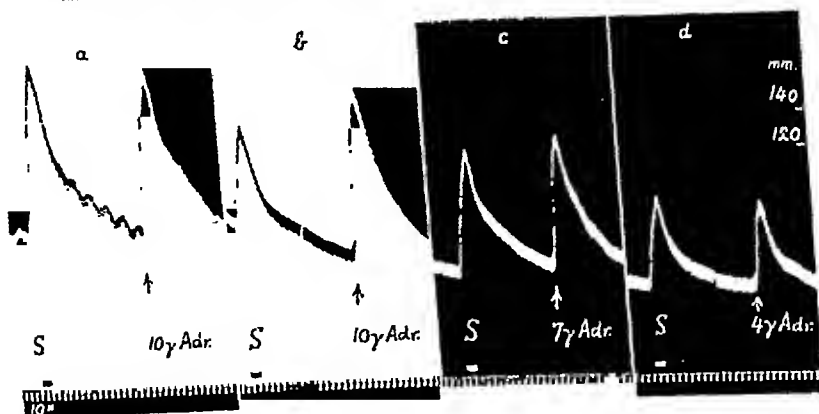


Fig. 9. Spinal cat, suprarenals excluded. (a) The pressor response to stimulation (S) of both splanchnic nerves (18 per sec. for 15 sec.) equals that of 0.01 mg. adrenaline; (b) 10 min. after 0.15 mg. adrenaline the splanchnic effect is reduced to half the size; after 20 min. (c) it equals 0.007 mg. adrenaline; after 30 min. (d) it equals 0.004 mg. adrenaline.

after periods which were longer the larger the dose; thus after 0.04 mg. the period was 20–25 min.; after 0.15 mg. the depression sometimes lasted over an hour or never recovered. In Fig. 8 the response of splanchnic stimulation is reduced to about half its former size after the injection of 0.08 mg. adrenaline. In order to see whether, after such an excessive vasoconstriction, the blood vessels failed to constrict or whether the ganglia failed to transmit the impulse, the pressor response to splanchnic stimulation was matched with that of a dose of adrenaline. In Fig. 9(a) splanchnic stimulation produced the same

pressor effect as 0.01 mg. adrenaline. Ten min. after the injection of 0.15 mg. adrenaline (*b*), the splanchnic effect was much reduced but the adrenaline effect was unchanged. Ten min. later (*c*), the splanchnic response showed a partial recovery and a rise of blood pressure equal to that of 0.007 mg. adrenaline was observed; but another 10 min. later (*d*) the splanchnic effect only equalled that of 0.004 mg. adrenaline; it did not return to its initial size. It was the rule to see this sequence of events when large doses of adrenaline were injected: after an initial depression the splanchnic response would show a partial recovery and then decline again. At the same time, the pressor effect

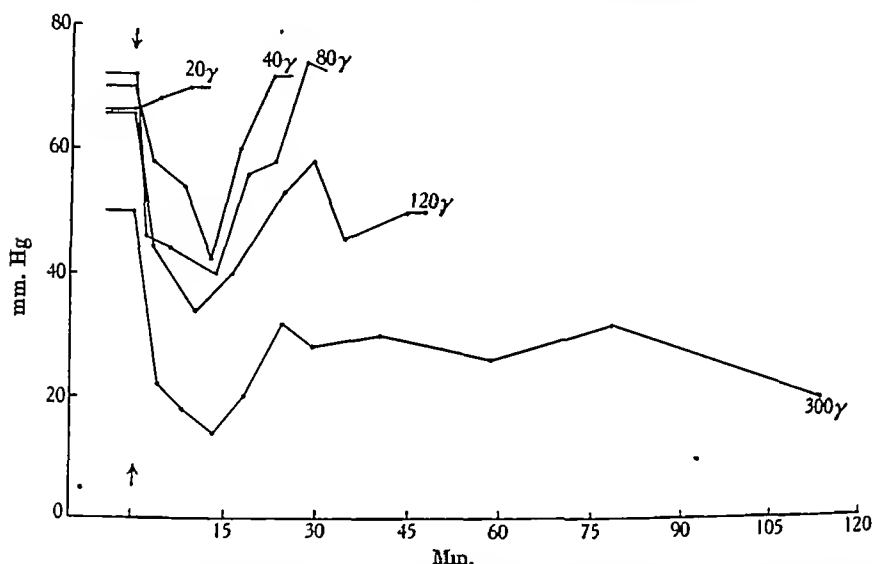


Fig. 10. Spinal cat, suprarenals excluded. The diagram shows the effect of five different doses of adrenaline on the response to splanchnic stimulation. Ordinates are pressor effects in mm. Hg of stimulating both splanchnic nerves. Abscissae are minutes after the adrenaline rise of blood pressure had disappeared.

of adrenaline was sometimes increased at first, then remained unchanged over a long period, and only in the final stages when the blood pressure became very low it was reduced by 10 or 20 %. The adrenaline response was never affected to such a degree as the response to splanchnic stimulation, which, after large doses of adrenaline, was regularly reduced to a quarter of its original size. It may be questioned whether this later depression was the result of the injection, but when large doses of adrenaline were not injected we never observed this decline of the vasoconstrictor response. Moreover, the depression was always accompanied by a fall in general blood pressure, presumably due to a depression at the sympathetic ganglia of the impulses proceeding to the muscles and the skin, a parallel depression to that we recorded when we stimulated the splanchnic nerves.

The course of the changes in the vasoconstrictor response is shown in Fig. 10, which represents the observations made in one experiment, in which adrenaline was injected in five different doses. Each point represents the actual rise in blood pressure produced by splanchnic stimulation. The injection of 0.02 mg. adrenaline increased the effect, whereas 0.04 mg. and 0.08 mg. depressed it. When 0.12 mg. was injected the response never returned to its former height, and a further lasting depression followed the injection of 0.3 mg. It should be added that similar results were obtained in cats anaesthetized with chloralose, though more adrenaline was needed to depress permanently the response to stimulation.

### DISCUSSION

It has been shown by three methods that the transmission of impulses through sympathetic ganglia is facilitated by small doses of adrenaline, but depressed by large ones. The facilitation by small doses is of interest since Stöhr has described chromaffine cells in sympathetic ganglia, and because it suggests a co-operation between adrenaline and acetylcholine at another site of cholinergic transmission. It was our observation of such a co-operation in the spinal cord which led us to look for it and to find it first at the neuromuscular junction in skeletal muscle and then in the sympathetic ganglion.

In two of the methods, electrical stimulation was applied to the preganglionic fibres, and the augmentation of the effect by small doses of adrenaline might have been due to a lowering of the threshold in the preganglionic fibres rather than to an effect on the ganglion. Bülbring & Whitteridge [1941] have observed that adrenaline lowers the threshold of the sciatic nerve to submaximal stimuli, and not to maximal stimuli. In the perfusion experiments submaximal stimuli were used, but in the experiments on spinal cats we applied maximal stimuli, so that changes in the preganglionic fibres were not involved. Moreover, when acetylcholine was used as a chemical stimulus to the ganglion, a similar augmentation by adrenaline was observed.

Marrazzi [1939a] showed that adrenaline depressed impulses through sympathetic ganglia, but he saw no augmentation. His failure to observe the augmentation may be due to the time after the adrenaline injection at which he took his records. Some of our results suggest that if he had made his observations over longer periods he would have seen augmentation following the depression. After the adrenaline injection we had to wait until its pressor action had subsided before recording the response to ganglionic stimulation. With doses from 0.01–0.02 mg. adrenaline we saw first a depression of the ganglionic response followed by an augmentation, the depression being present from 5 to 15 min. after the injection, and the augmentation appearing after 20 min. It is probable that this sequence occurs also after smaller doses but at shorter intervals after the injection. It should be mentioned that, while in our experiments with acetylcholine we regularly observed the depressing action

of adrenaline, we had much more difficulty in observing the augmenting action. This was not so when we stimulated the splanchnic nerves. A steady infusion of adrenaline had no depressant action, even though the amounts were as large as 0.008 mg./kg./min., as long as the infusion continued. Depression was, however, observed when such an infusion was stopped or after the injection of a single large dose.

The depression of ganglionic transmission by adrenaline has been discussed by Marrazzi [1939b] as a mechanism having a beneficial effect, whereby adrenaline can check excessive sympathetic activity. He says: 'Adrenaline liberated by the augmented splanchnic impulses prolongs and greatly enhances sympathetic activity. If this tends to become excessive, thereby partially defeating its purpose, the concentration of adrenaline in the blood rises to a level sufficient to produce ganglionic inhibition, and thus decreases the sympathetic discharge by obstructing the passage of impulses from the pre- to the post-ganglionic neurones.' It may be that in certain circumstances this effect is beneficial, as Marrazzi thinks, but there is also another possibility. We believe that it may play a part, in the production of shock. Freeman, Freedman & Miller [1941] have shown that the infusion of adrenaline into dogs caused a steady decline of blood pressure, which continued after the infusion was stopped, and led to death. According to our results, the doses they used were certainly great enough to cause ganglionic depression after the infusion was discontinued, and we think that this depression must have been partly if not wholly responsible for the fatal fall in blood pressure. Probably there are many workers who have observed, as we have, that when an adrenaline infusion is stopped the blood pressure falls below the initial level, and may not return. It is of course a well-known observation that after a large single dose, the pressor effect is followed by a temporary fall.

The view that excessive sympathetic activity is a cause of shock has many opponents, who quote the evidence of Prohaska, Harms & Dragstedt [1937] that continuous adrenaline infusion of amounts up to 0.003 mg./kg./min. into dogs did not cause death from shock. These amounts were sufficient to cause hypertension, though they were not so great as those used by Freeman *et al.* which were from 0.003 to 0.016 mg./kg./min. Our own experiments suggest that the sudden liberation of large amounts of adrenaline is more likely to produce permanent circulatory damage than the steady infusion of smaller amounts. Under the influence of an overwhelming sensory stimulus, there can be no doubt that a liberation of a large amount of adrenaline occurs. After some accidents clinicians describe an early stage of collapse with low blood pressure, and then, after a period of recovery, a later stage in which the blood pressure falls again. When a large dose of adrenaline has been injected we have observed changes in the response to splanchnic stimulation which correspond with this chain of events as shown in Figs. 8-10.



## SUMMARY

1. Evidence has been obtained by three different methods that adrenaline in small amounts augments the transmission of impulses in sympathetic ganglia, and in large amounts depresses it. This action of adrenaline has been observed in dogs in which the sympathetic ganglia were perfused by one circulation and the responding organ, the vessels of the hindleg, by a second.

2. In atropinized spinal cats adrenaline affects the ganglionic action of acetylcholine, the pressor effect of which is augmented by small doses and depressed by large doses of adrenaline.

3. When adrenaline is infused at constant rate into a spinal cat the pressor response to splanchnic stimulation is increased; the injection of single large doses however depresses the response. This depressant action, which may be permanent and is accompanied by a fall in general blood pressure, is discussed in its possible relation to shock.

We wish to express our thanks to Mr H. W. Ling for his assistance with the perfusion experiments.

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## MINERAL METABOLISM ON DEPHYTINIZED BREAD

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Although by the year 1925 oatmeal and wheat had been clearly demonstrated by Mellanby to have rachitogenic properties, the discovery was grudgingly admitted to be true by a world which relied largely on cereals for its nutrition. E. & M. Mellanby found that the rachitogenic agent was destroyed by boiling the cereals with 1% HCl, and also by germination [M. Mellanby, 1929], but they were not able at that time to identify it. Bruce & Callow [1934], following up an observation of Steenbock, Black & Thomas [1930], showed that cereals were rachitogenic for rats on high-calcium low-phosphorus diets because a large part of the phosphorus in them was present as inositol hexaphosphoric, or phytic, acid, and hence was not so freely utilized as inorganic phosphate would have been. These findings at once received recognition. McCance & Widdowson [1935] then showed that phytate phosphorus was comparatively unavailable to man, a fact which had really been demonstrated by balance experiments on children as long ago as 1916. Ahlqvist [1916], however, who made these observations, had not designed her experiments for this purpose, and their significance seems to have escaped her. Nevertheless, she found that when 194 mg. of phytic acid phosphorus were added to diets which contained an average of 1510 mg. of phosphorus per day, the percentage of the intake absorbed fell from 65 to 58. The magnitude of this fall suggests that these children absorbed none of the phytic acid phosphorus, but this is probably an over-statement of the facts. Bruce & Callow [1934] also carried out some inconclusive experiments with low-calcium diets. They pointed out, however, that phytic acid had a calcium salt 'at least as insoluble as calcium phosphate', and that, therefore, it might be expected to lower the available calcium in such diets, possibly even to deficiency levels, by binding the metal in a precipitated form. McCance [1934] enlarged upon this property of phytic acid, and Mellanby recognized at once that it was likely to be the important one in the genesis of canine and human rickets, which are due to calcium rather than phosphorus deficiencies. As late as 1937, however, the considered opinion of the American 'Council on Foods' still was that 'there is no good evidence for the existence of a decalcifying factor in cereals'. Harrison & Mellanby [1939]

took up the matter experimentally, and showed that diets could be made rachitogenic for puppies by incorporating sodium phytate into them. Mellanby [1941] has since demonstrated that as oatmeal is boiled with dilute HCl, the phytic acid in it is slowly destroyed, and its rachitogenic properties gradually diminish. Phytic acid, therefore, may be accepted as the ingredient which makes oatmeal so detrimental to the growing bones and teeth of dogs.

Returning to this problem because of some chance observations which had been made in an experimental study of rationing, McCance & Widdowson [1942] showed that healthy men and women absorbed calcium and magnesium much less readily from brown than from white bread dietaries. The subjects obtained 40-50% of their total calories from the staple cereal, the remainder from an ordinary mixed diet. It was considered that the reduced absorption was probably due to the large quantities of phytic acid in brown bread, and this view was enhanced by the fact that the effects of brown bread on mineral metabolism could be reproduced by adding sodium phytate to white bread. Nevertheless, the fact still remained that brown bread had a laxative action, and there was as yet no proof that this was not playing some part in hindering calcium absorption [Ascham, 1930-1; Aub, Tibbetts & McLean, 1937; Bloom, 1930]. It was felt that phytic acid could be even more definitely incriminated if experiments could be carried out with brown bread from which the phytic acid had been removed, and this investigation will now be described.

### METHODS

*Subjects.* There have been six subjects, three men and three women. One man, E.B., and one woman, A.M., had taken part in the previous investigation and were described in the report of it [McCance & Widdowson, 1942]. N.J., one of the new men, was a University teacher, aged 27 and weighing 142 lb., and the other, N.C., was a postgraduate student, aged 21, weight 140 lb. Of the new women, H.E. was a research student, aged 21, weight 118 lb., and W.Y. a doctor, aged 32, with a weight of 147 lb.

*Number and order of the experiments.* Each subject has carried out four experiments, a white bread control, a brown bread control, and experiments with two different kinds of dephytinized brown bread. The dietary and other technical arrangements were the same as those reported by McCance & Widdowson [1942], and, as before, the flour under investigation furnished 40-50% of the subjects' calories. The experiments began in September and ended just before Christmas. The first three lasted for 3 weeks and contained three analytical periods each of 1 week in length, but the December experiments only lasted a fortnight, for the results were a foregone conclusion by that time and several of the subjects were anxious to be free for Christmas. The preliminary periods were 3 days in length, and the usual after-periods were provided. Full metabolic discipline was rigidly enforced, including the pro-

hibition of all tooth pastes [McCance & Widdowson, 1942]. In order to prevent seasonal or intrinsic fluctuations in calcium absorption from influencing the results [McCance & Widdowson, 1943], the subjects did not all do the experiments in the same order. Two began with the white bread control, two with the brown bread control and two with a dephytinized preparation. The later experiments were similarly arranged.

*Preparation and properties of the breads.* A 'C roll' white flour, i.e. one of very low extraction, and bran from the same grist, ground to pass through a  $\frac{1}{32}$  in. mesh, were obtained from the Research Association of British Flour Millers, and these formed the basis of all the flours used in these experiments. The white bread was made from the white flour, with yeast as the raising agent. The brown bread was made from a reconstituted flour containing 83 parts of white flour and 17 parts of bran. This bread was baked with sodium bicarbonate and acid potassium tartrate in order to avoid hydrolysis of the phytic acid [Widdowson, 1941] and contained about 0.1% of phosphorus in the form of phytic acid.

The other experimental breads were made in the following way. Bran was dephytinized enzymically by incubating it at 50° C. with 10 times its weight of water for 6 hr. at pH 4.5. The pH was adjusted by adding HCl, and at the end of the period of incubation this was neutralized with NaOH and the bran filtered off through a closely woven cotton cloth on a large Buchner funnel. The bran was then spread out to dry on a sheet of the same cloth tied over wires on a wooden frame set over an electric fire. The enzymic process broke down the phytic acid into inositol and inorganic phosphates. These, and the mineral ions with which the phytic acid had originally been combined, tended to pass into the liquid, as also did some of the more soluble nitrogenous and other organic compounds in the original bran. Consequently, the amount of dried bran recovered from the dephytinizing process was usually about 70% of the weight taken at the beginning. Of the water added to the bran, about 85% was usually recovered in the filtrate. The remainder stayed with the bran and was removed by volatilization as the bran was dried. Table 1 shows the composition of the original bran and the partition of its constituents by the dephytinizing process. Apart from the solids, nitrogen and phosphorus, to which reference has already been made, it will be noted that the metallic ions passed into the filtrate in very different amounts, and that the order of solubility was potassium, magnesium, calcium and iron. Practically none of the last left the bran. Whereas, therefore, the original bran contained more than enough phytic acid to combine with all the calcium, magnesium and iron, the dephytinized and demineralized bran did not, and the physico-chemical properties of magnesium and calcium phytates [McCance & Widdowson, 1942] indicate that most of the magnesium was in combination with some other acid radicle.

TABLE 1. The composition of bran and of its dephytinized products

*	Composition of the original bran g./1000 g.	Distribution of the solids and chemical elements in 1000 g. of bran between air-dried dephytinized bran and the filtrate separated from it	
		Dephytinized bran g.	Filtrate g.
Solids	1000	694	306
Total nitrogen	23.6	14.0	9.6
Total phosphorus	12.4	3.2	9.2
Phytic acid phosphorus	11.0	0.92	tr.
Calcium	1.1	0.75	0.35
Magnesium	5.8	1.6	4.2
Potassium	13.0	3.0	10.0
Iron	0.137	0.134	tr.

\* Both the original bran and the air-dried dephytinized product contained small amounts of water, but these have been neglected in constructing this table.

The processed bran was used to reconstitute a brown flour, and since the incubation and filtration not only hydrolysed the phytic acid but also removed a large proportion of the resulting phosphates, potassium and magnesium, the bread so baked may best be described as a dephytinized and demineralized brown bread. The amount of the processed bran to be added to the white flour was determined by the weight which it had lost during its dephytinization. Thus if 17 g. of natural bran were used in order to reconstitute 100 g. of 'brown' flour, and if 100 g. of this bran lost 30 g. during dephytinization, then 13 g. of the product were used for the reconstitution of 100 g. of the dephytinized and demineralized brown flour. In order to study the effects of dephytinization without demineralization the liquid filtrate in the correct proportions was incorporated into the diets. Some of it was used to make up the dough from which the bread was to be baked, and the remainder was measured out in the correct quantities and drunk by the subjects as they ate the bread. The approximate compositions of the three kinds of bread and the bread-filtrate combination are given in Table 2. There are some rather interesting points about these figures. The only important difference between the brown and the dephytinized brown is the change of the phytic acid phosphorus to inorganic phosphorus, but there may have been changes in the state of combination of all the ions following the enzymic processes. The demineralized bread differed from the dephytinized bread in containing rather more than one-third as much phosphorus and magnesium and about four-fifths as much calcium. It is suggested that the magnesium was present as magnesium phosphate, maintained in solution during incubation of the bran at pH 4.5, but precipitated in, or with, the dephytinized bran when the NaOH was added before filtration. The white bread contained nearly as much phosphorus but considerably less magnesium than the demineralized bread.

TABLE 2. Composition of the four experimental breads

	Brown	Dephytinized brown (including the correct amount of filtrate draught)	Dephytinized and demineralized brown	White
Water, g./100 g.	36	35*	35	32
Total nitrogen, g./100 g.	1.52	1.52	1.44	1.56
Protein, g./100 g. ( $N \times 6.0$ )	9.1	9.1	8.6	9.4
Total phosphorus, mg/100 g.	206	206	75	66
Phytic acid phosphorus, mg/100 g.	99	13	13	0
Calcium, mg/100 g.	25	25	19	15
Magnesium, mg/100 g.	85	85	30	17
Iron, mg/100 g.	2.3	2.3	2.3	1.0

\* The water in the filtrate draught is not included in this table.

It is rather useful in considering the metabolic results to bear in mind the approximate proportions of the total dietary calcium, phosphorus and magnesium supplied by the three different breads and the bread-filtrate combination. The figures for the men are given in Table 3, and it will be

TABLE 3. Approximate percentages of the men's total dietary calcium, magnesium and phosphorus supplied by the four 'breads'

	Brown	Dephytinized brown (with filtrate draught)	Dephytinized and demineralized brown	White
Calcium	30	30	25	20
Magnesium	74	74	57	33
Total phosphorus	70	70	45	40

appreciated at once that most of the calcium came from foods other than the bread, and that all four breads supplied roughly the same percentage of the dietary calcium. 70% of the dietary magnesium and phosphorus was provided by the brown and the dephytinized brown bread; much less was provided by the demineralized bread and only 33% of the dietary magnesium by the white bread. Consequently, the magnesium and phosphorus results are more difficult to interpret than the calcium findings, and that for two reasons. First, the absorption of the magnesium and phosphorus present in the brown breads weighted the figures for the total absorptions much more than did the absorptions of these two minerals from white bread. Secondly, since the basal diet did not change from one experiment to the next, the total intakes of magnesium and phosphorus fell greatly on passing from the brown and dephytinized brown breads through the demineralized bread to white.

The reconstituted brown bread and the dephytinized and demineralized bread were good both in appearance and taste. The filtrate was slightly salty but, flavoured with lemon essence, it was not difficult to drink.

## METABOLISM RESULTS

## Calcium

Table 4 shows the calcium intakes and absorptions of the six subjects on the four different diets. It will be noted that the intakes were a little higher on the brown bread and the dephytinized bread than they were on the demineralized and white breads, but that the intakes were of the same order and roughly comparable throughout. It will next be noted that on any given

TABLE 4. The intakes and absorptions of calcium

Type of bread	Intake mg./day	Subject E.B.		Intake mg./day	Subject H.E.	
		Absorption mg./day	% of intake		Absorption mg./day	% of intake
Brown	550	89	16	522	57	11
Dephytinized brown	590	231	39	568	169	30
Dephytinized and demineralized brown	472	236	50	490	192	39
White	488	250	51	478	219	46
Subject N.C.				Subject A.M.		
Brown	558	74	13	558	7	1
Dephytinized brown	602	279	46	606	120	20
Dephytinized and demineralized brown	559	327	58	480	150	30
White	505	232	46	443	142	32
Subject N.J.				Subject W.Y.		
Brown	610	37	6	575	23	4
Dephytinized brown	662	67	10	633	123	19
Dephytinized and demineralized brown	593	123	21	512	149	28
White	522	111	21	552	183	33

diet the subjects tended to have absorptions very different from one another, even if their intakes were nearly the same. E.B. and N.J. make an excellent contrast in this respect, for N.J. consistently absorbed very much less calcium, although he always had a somewhat larger intake. Nevertheless, the same alterations in diet, i.e. the same changes in external conditions, changed the absorptions of all the subjects in the same direction. This is a satisfactory feature of the results. Finally, and this is the most important point demonstrated by the table, the absorptions of calcium were all at their worst on brown bread. In fact, all the subjects had negative balances on these diets. The calcium absorptions were all improved by the hydrolysis of the phytic acid, but further improvement took place when the phosphates were removed. Four of the subjects absorbed calcium slightly better when white replaced the dephytinized and demineralized bread. One subject did not, and on the whole the differences between the calcium absorptions on these two diets were small. The interest of this observation lies in the fact that the dephytinized and demineralized bread was highly laxative. Indeed, as might have been anticipated from the work of Falcon-Lesses [1929-30] on rats, the processed was really as

laxative as the original bran. The average weights of the fresh faeces passed daily were 116 g. on white bread, 209 g. on the dephytinized and demineralized bread, 226 g. on the dephytinized bread, and 224 g. on the brown bread. Comparison of these faecal weights with the calcium absorptions (Table 4) shows that it was not the increased bulk of the faeces on brown bread diets which made the absorptions of calcium so much lower than they were on white. The poor absorptions, therefore, of calcium from brown and dephytinized brown bread must be attributed to the specific action of the phytates and phosphates [McCance & Widdowson, 1942]. This conclusion is in keeping with the findings of Aub *et al.* [1937] in human subjects, and substantially, also, with the work of Ascham [1930-1] on dogs and of Bloom [1930] on rats.

### Magnesium

Table 5 shows the intakes and absorptions of magnesium on the four different diets. Some of the results are like those of calcium and are equally conclusive, and a comparison of the absorptions from the brown and from the dephytinized brown bread diets shows that phytates interfered with magnesium

TABLE 5. The intakes and absorptions of magnesium

Type of bread	Intake mg./day	Absorption mg./day	Absorption % of intake	Intake mg./day	Absorption mg./day	Absorption % of intake
		Subject E.B.			Subject H.E.	
Brown	720	120	17	650	163	25
Dephytinized brown	704	214	30	524	152	28
Dephytinized and demineralized brown	370	143	39	352	124	35
White	307	140	45	270	160	59
		Subject N.C.			Subject A.M.	
Brown	754	47	6	592	140	24
Dephytinized brown	764	208	27	564	154	27
Dephytinized and demineralized brown	505	207	41	318	126	39
White	320	163	51	230	109	47
		Subject N.J.			Subject W.Y.	
Brown	740	166	22	638	176	28
Dephytinized brown	748	207	28	618	217	35
Dephytinized and demineralized brown	480	177	37	378	176	46
White	324	164	50	341	193	57

as they did with calcium absorption. There are, however, some interesting differences between the magnesium and the calcium results. The first is the great fall in the magnesium intake brought about by the demineralization of the bran. Such a fall may have combined with the removal of phosphates in helping to improve the percentage absorptions on the demineralized diets, for there is some evidence that as magnesium intakes rise the percentage absorptions tend to fall [Tibbetts & Aub, 1937]. Secondly the magnesium in the demineralized bran (see Table 1) was less readily absorbed than the magnesium in the white



bread diets. Taking the intakes and subjects as a whole, the differences in the amounts ingested on these two diets were scarcely enough to have accounted for this. There are good reasons, moreover, for thinking that it was not due to the laxative properties of the demineralized breads. It can hardly have been caused by the traces of residual phytic acid, for calcium, which forms a more insoluble phytate than magnesium, was almost as well absorbed from the demineralized as from the white bread diets. It may be that the magnesium in this dephytinized and demineralized bread was combined with phosphate, and that the two ions were mutually preventing each other's absorption (see later), but it is possible that the magnesium in the bran was in some unknown but stable combination. Lithium seems to form such salts [Kent & McCance, 1941].

### Phosphorus

Table 6 shows the phosphorus absorptions of the six subjects on the four different kinds of bread. The change from brown to dephytinized brown much improved the absorptions of all the subjects, and the average rose from 48 to

TABLE 6. Intakes and absorptions of phosphorus (including phytic acid phosphorus)

Type of bread	Intake mg./day	Absorption mg./day	Absorption % of intake	Intake mg./day	Absorption mg./day	Absorption % of intake
		Subject E.B.			Subject H.E.	
Brown	1940	900	46	1650	819	50
Dephytinized brown	1900	1230	65	1550	980	63
Dephytinized and demineralized brown	1200	810	68	1110	724	66
White	1260	923	73	1090	875	80
		Subject N.C.			Subject A.M.	
Brown	1920	840	44	1490	680	46
Dephytinized brown	2070	1400	68	1500	914	61
Dephytinized and demineralized brown	1450	1010	69	890	560	63
White	1290	1010	78	880	620	70
		Subject N.J.			Subject W.Y.	
Brown	2050	965	47	1850	980	53
Dephytinized brown	2030	1250	61	1720	1080	63
Dephytinized and demineralized brown	1440	890	62	1080	655	61
White	1220	847	70	1180	842	71

64% of the intakes. This was expected, for phytic acid phosphorus was already known to be less readily absorbed than inorganic phosphorus [McCance & Widdowson, 1942]. It is of some interest to refer here to a remark of Blatherwick & Long [1922] that the phosphorus of washed bran is unavailable to man, whereas a certain part of the phosphorus of unwashed bran is assimilated. It is easy to see now that the large amount of phytic acid phosphorus in bran was the basis for this observation, although in the light of recent work their statement must be regarded as somewhat of an exaggera-

tion. On the demineralized bread the intakes fell to a little over half what they had been before, but the percentage absorptions remained virtually unchanged. The intakes on demineralized bread and on white bread were about the same, but the absorptions on white bread were better. The absorptions averaged 65 and 74 % of the respective intakes. The small difference in the amount of phytic acid phosphorus in the two breads is not quite enough to account for the whole of this further, although admittedly smaller, improvement in absorption. If, however, the magnesium and phosphate ions in the demineralized bran were combined with each other and mutually preventing each other's absorption, then the absence of bran and consequently the smaller magnesium intakes on the white bread diets probably contributed to the improved absorptions of phosphorus.

### DISCUSSION

The results of these experiments speak for themselves and they require little discussion. Taken in conjunction with the results of the experimental work previously reported [McCance & Widdowson, 1942], they leave little room for doubt that phytic acid is the agent in whole wheat primarily responsible for the poor absorptions of calcium and magnesium. They confirm also that the phosphorus in phytic acid is absorbed less freely than inorganic phosphorus. They show, however, that mere hydrolysis of phytic acid will not permit calcium and magnesium to be absorbed as freely from brown flour as from white. Some of the products of hydrolysis also interfere to some extent, and the whole trend of work on calcium and phosphorus metabolism and the present results themselves suggest that the inorganic phosphates are responsible. It is evident, therefore, that even if the processing of brown flour could so be organized that all the phytic acid were hydrolysed [Widdowson, 1941], the resulting bread would still compare unfavourably with white bread so far as its effects upon calcium absorption were concerned. This is not to say that wholemeal bread is a worse food for man than white bread. The evidence generally is that it is better, except for the fact that it contains so much phytic acid. The addition of calcium to the brown flour would at once correct this defect.

The whole of this work on human metabolism should be regarded as confirming, expanding and in part explaining the pioneer work of the Mellanbys on puppies [Mellanby, 1925; M. Mellanby, 1929; Harrison & Mellanby, 1939]. It is satisfactory to have been able to extend their work to the species to which it was always intended to apply, and to have been able to show that the growing puppy, deprived of vitamin D, was a better indicator of what went on in the human intestine than many people supposed.

## SUMMARY

1. The calcium, magnesium and phosphorus absorptions of three men and three women were studied on diets in which 40-50% of the total calories were supplied in the form of:

- (a) a 'brown' flour reconstituted from white flour and bran;
- (b) a flour mixture similar to (a) in which the phytates had been hydrolysed enzymically to inorganic phosphates and inositol;
- (c) a mixture of white flour and bran from which nearly all the phytates and most of the products of hydrolysis had been removed;
- (d) white flour.

The laxative properties of (a), (b) and (c) were approximately the same.

2. The calcium absorptions were worse in (a) than (b) and in (b) than (c), but almost the same in (c) and (d).

3. The magnesium absorptions improved progressively in passing from (a) to (d).

4. The phosphorus absorptions were worse in (a) than in (b) or (c), and a little worse in (b) or (c) than in (d).

Miss B. K. Alington has played a very large part in making this investigation a success. Those who have taken part in similar studies will appreciate how much has been due to the good will and self-discipline of the six subjects. The work has largely been financed by the Medical Research Council, and E. M. W. is in the whole-time service of the Council.

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## THE OSMOTIC PRESSURE OF FOETAL HORSE-SERUM ALBUMIN

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for Medical Research, N.Y.*

(Received 22 April 1942)

In a previous paper [McCarthy, 1938] it has been shown that for the same concentration the serum proteins of the foetal sheep give a higher osmotic pressure than those of the maternal sheep. Since only a part of this effect could be accounted for by the albumin/globulin ratios, it was suspected that the foetal-serum albumin may be different.

In order to obtain further information it is desirable to study individual proteins, and for this purpose horse serum is preferable because the horse-serum albumin is readily crystallized.

In this work it was found that new-born foal serum contains an albumin which is easily crystallized by the method of Adair & Robinson [1930a].

### METHOD

About 100 c.c. of serum was prepared from the umbilical cord blood of a new-born foal. A preparation of twice crystallized albumin was obtained by the rapid method described by Adair & Robinson [1930a]. A series of measurements of osmotic pressure was made on the foal-serum albumin, using the same buffer mixture, and methods similar to those of Adair & Robinson [1930b]. Osmometers were set up at 0° C. until equilibrium was attained. Determinations of the capillarity correction and density of the protein solutions, and of the density and pH of the dialysate were made subsequently. The protein concentration was calculated from nitrogen estimated by the gasometric micro-Kjeldahl method [Van Slyke, 1927]. The factor used for calculation of the protein concentration from nitrogen estimation was based on the figure for horse-serum albumin 15.6% N [Adair & Robinson, 1930a].

### RESULTS

The results are shown in Table 1, in which  $P$  is the osmotic pressure reduced to mm. Hg at 0° C., and  $C$  represents the concentration expressed as grams of protein per 100 c.c. of solution.

<sup>1</sup> Holder of a grant from the Medical Research Council of Ireland.

Fig. 1 shows the data recorded in the table together with those obtained on horse-serum albumin by Adair & Robinson [1930b]. In comparing the two sets of observations it will be seen that at the higher concentrations, where

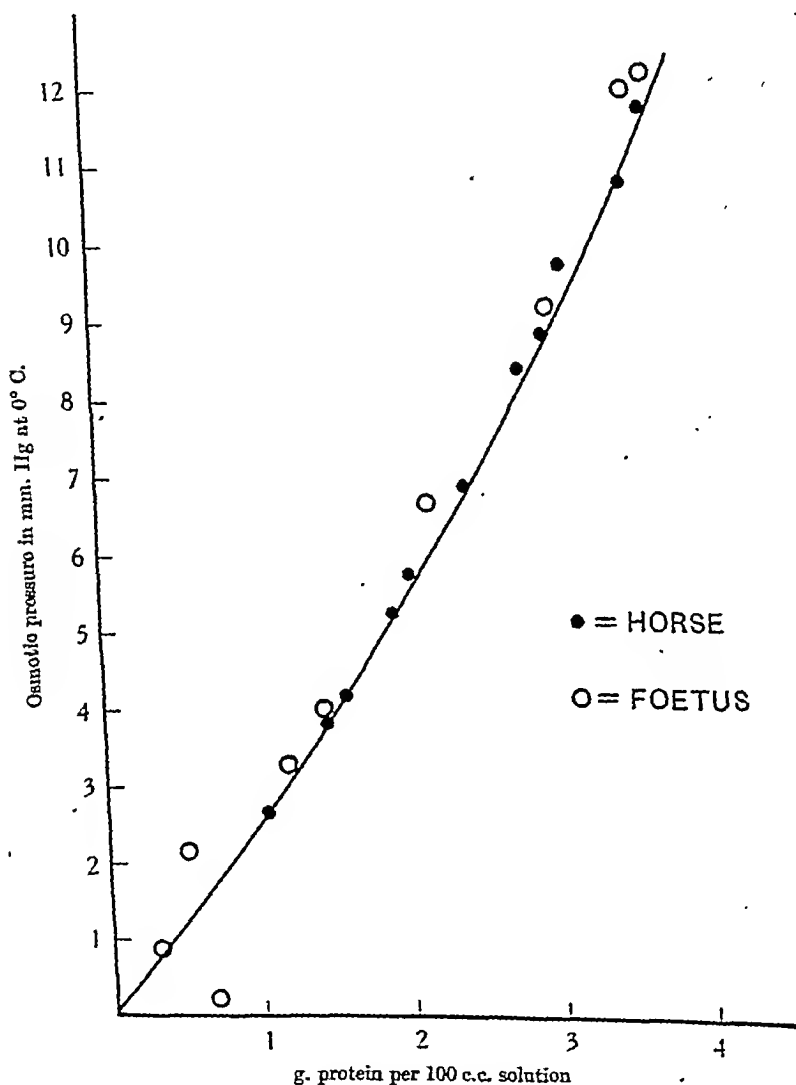


Fig. 1. The curve is constructed from the data of Adair & Robinson [1930b] on horse-serum albumin.

the measurements are more accurate, the osmotic pressure of foetal-serum albumin tends to exceed that of the adult. These differences are small, being from  $2\frac{1}{2}\%$  to  $4\frac{1}{2}\%$ , and, considering the range of error, are hardly significant. Differences of this order were observed by Adair & Robinson [1930b] in com-

TABLE 1. Osmotic pressure of foetal-serum albumin in mm. Hg at 0° C.

Exp. no.	P	C	pH glass electrode	Density protein solution	Density dialysate
1	11.88	3.56	7.18	—	—
2	9.16	3.02	7.26	1.01706	—
3	6.81	2.19	7.26	1.01496	1.00842
4	4.02	1.45	7.26	—	—
5	0.21	0.68	7.26	1.00996	—
6	2.17	0.52	7.26	1.01121	1.00880
7	12.11	3.70	7.26	—	—
8	3.29	1.20	7.26	—	—
9	0.89	0.31	7.26	1.00978	1.00813

parisons of normal horse serum obtained from different sources. It may be inferred that if the molecular weight of the adult-serum albumin is 72,000, the molecular weight of foetal-serum albumin is 69,000. This figure must be regarded as a provisional value, because the precision of the observations on the more dilute solutions is not sufficient for a determination of the molecular weight by extrapolation as described by Adair & Robinson [1930*b*].

### SUMMARY

Crystalline serum albumin was prepared from foetal-horse blood. Osmotic pressure measurements revealed no striking differences in the properties of foetal and adult horse-serum albumin. If the albumins of foetal and maternal sera are the same, as seems probable at least in the case of the horse, the high osmotic pressure per unit of protein observed in foetal-sheep serum must be attributed to the globulin fraction.

I wish to express my thanks to Dr D. D. Van Slyke for his kindly interest and laboratory facilities, and to Mr G. S. Adair, for his helpful criticism. I also wish to thank Dr P. E. Howe and Dr Imogen Earle for supplying the foetal serum.

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THE POSTERIOR PITUITARY PRINCIPLES OF A SPECIES  
OF REPTILE (*TROPIDONOTUS NATRIX*) WITH SOME  
REMARKS ON THE COMPARATIVE PHYSIOLOGY  
OF THE POSTERIOR PITUITARY  
GLAND GENERALLY

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(Received 15 May 1942)

There is now general agreement that the posterior pituitary secretion of mammals aids in the conservation of body water by producing an increase of the tubular reabsorption of water, and thus leads to the production of a hypertonic urine. The physiology of posterior pituitary action in other classes of vertebrates has been neglected mainly because lower vertebrates, with the exception of birds, are unable to prepare a hypertonic urine. The mammalian mechanism of posterior pituitary action can, therefore, not apply.

However, there is one group of lower vertebrates for which a physiological importance of the posterior pituitary secretion for the regulation of their water balance has been suggested. Small doses of posterior pituitary extracts injected into frogs or other amphibians produce a temporary increase of body water. Steggerda [1937], working with several species of amphibians, showed a definite correlation between the magnitude of this effect and the natural habitat of the species. The more terrestrial the species the bigger the effect of a given dose of posterior pituitary extract on its water balance. There is ample evidence to prove that this effect is produced, partly at least, by an extrarenal mechanism, viz. by a change in the permeability of the amphibian skin [Brunn, 1921; Steggerda, 1931; Novelli, 1936].

Bearing these mechanisms in mind, how is one to comprehend a physiological action of the posterior pituitary gland in reptiles, i.e. in a class of vertebrates which are unable to excrete a hypertonic urine [Burian, 1910; Smith, 1932] and whose habitat and skin structure excludes an extrarenal mechanism similar to that of amphibians? There is a possibility that the posterior pituitary gland does not take part in the regulation of the water metabolism of reptiles. It was, therefore, of interest to determine whether the reptile pituitary gland contained any considerable amounts of those posterior lobe activities which are supposed to be concerned with the water metabolism of other vertebrate

classes. Herring [1913] reported the presence of a pressor principle in extracts of tortoise pituitary glands, but no demonstration of the antidiuretic and the 'water-balance' activity of reptile pituitary extracts has yet been made.

### METHODS

*Assay of antidiuretic activity.* Intravenous injections into unanaesthetized rabbits were employed. The intravenous injection of saline or, in other words, an intravenous injection as such does not interfere with the normal water

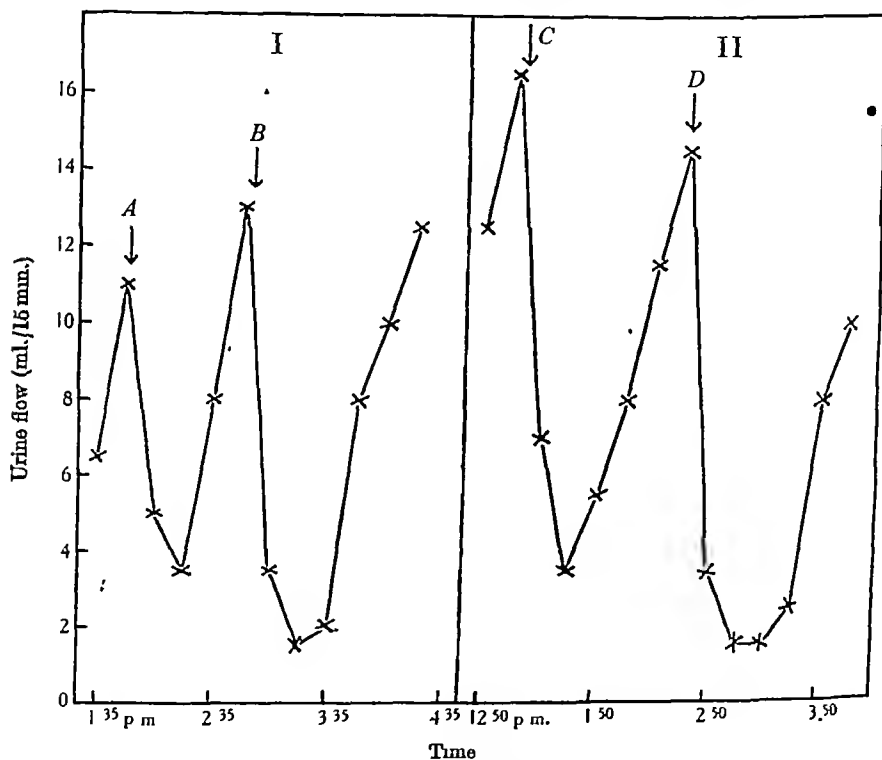


Fig. 1. Estimation of antidiuretic activity of grass-snake pituitary gland. Grass-snake no. 4, 50 g. ♂. I=rabbit no. 71. At 10 00 a.m. and at 12.10 p.m. 5% of body weight of water by stomach tube. A=1 0 mU. pitressin i.v. B=2 0 vol. % of grass snake pituitary gland extract i.v. II=rabbit no. 89. At 10 04 a.m. and at 12 09 p.m. 5% of body weight of water by stomach tube. C=0 75 vol. % of grass-snake pituitary gland extract i.v. D=1 0 mU. pitressin i.v. The antidiuretic activity of the grass snake pituitary extract was, therefore, equivalent to more than 50 mU. and to less than 130 mU. pitressin.

diuresis of a rabbit provided that care is taken not to excite the animal. To avoid excitement and struggling, it was found best to inject the rabbits without the help of a box or the hands of an assistant. Varying amounts of the extract of one reptile pituitary gland were matched with the response to a



standard dose of pitressin. Several rabbits were used to estimate the potency of any one grass-snake pituitary extract. Further details of the assay are given in the 'legend' to Fig. 1.

*Assay of (amphibian) water-balance activity.* English frogs (*Rana temporaria*) of an average weight of 20 g. were used. All experiments with frogs were done between April and September. The evening before the experiment started, each frog was placed in a covered beaker and immersed in tap water. Changes of weight of frogs kept under these conditions were small and inconsistent. Cross tests were used to compare the effect of any preparation on the water uptake of a series of frogs. Injections were made into a ventral lymph sac. The volume of each injection was made up to 0.5 c.c. Weighings were made 1 hr. before the injection and subsequently at hourly or half-hourly intervals. Before each weighing the frogs were carefully dried and the bladder thoroughly emptied. Care was taken to perform the cross tests at approximately the same temperature. However, the method of assay cannot be considered very accurate, and the results obtained should be regarded as indicating an order of magnitude rather than as precise estimations of the water-balance activity. The unit of water-balance activity used in this paper was defined as the amount of (frog) water-balance activity contained in 0.5 mg. of the international (mammalian) standard powder. It should be noted that Boyd and his co-workers [Boyd & Mack, 1940] use a different unit of water-balance or water-retention activity.

*Assay of pressor activity.* Spinal cats were used.

*Preparation of grass-snake pituitary extracts.* A series of fifteen English grass-snakes (*Tropidonotus natrix*), comprising both sexes, were used for the experiments presented. The animals were killed by decapitation, the whole pituitary gland and the parts of the brain proximal to the gland were removed and acid extracts were prepared without delay. The details of extraction are given in a previous paper [Heller, 1941*a*]. Control extracts of pieces of indifferent brain tissue were prepared in an identical manner.

The commercial (mammalian) posterior pituitary extracts employed were pitressin (Messrs Parke, Davis & Co.) and posterior pituitary extract (B.D.H.).

## RESULTS

*The antidiuretic activity of grass-snake pituitary extracts.* Preliminary 'range-finding' experiments are not quoted. The experiment shown in Fig. 1 is included in Table 1. It will be noted that the figures given for the antidiuretic activity of the snake posterior glands lie in a comparatively narrow range. (Six out of eight glands showed an average antidiuretic hormone content equivalent to between 75 and 100 mU. pitressin.) Figures of hormone content calculated per 100 g. of body weight are more dissimilar. However, they indicate an average antidiuretic hormone content which is higher than that of birds and amphibians but lower than that of mammals [Heller, 1941*a*].

classes. Herring [1913] reported the presence of a pressor principle in extracts of tortoise pituitary glands, but no demonstration of the antidiuretic and the 'water-balance' activity of reptile pituitary extracts has yet been made.

### METHODS

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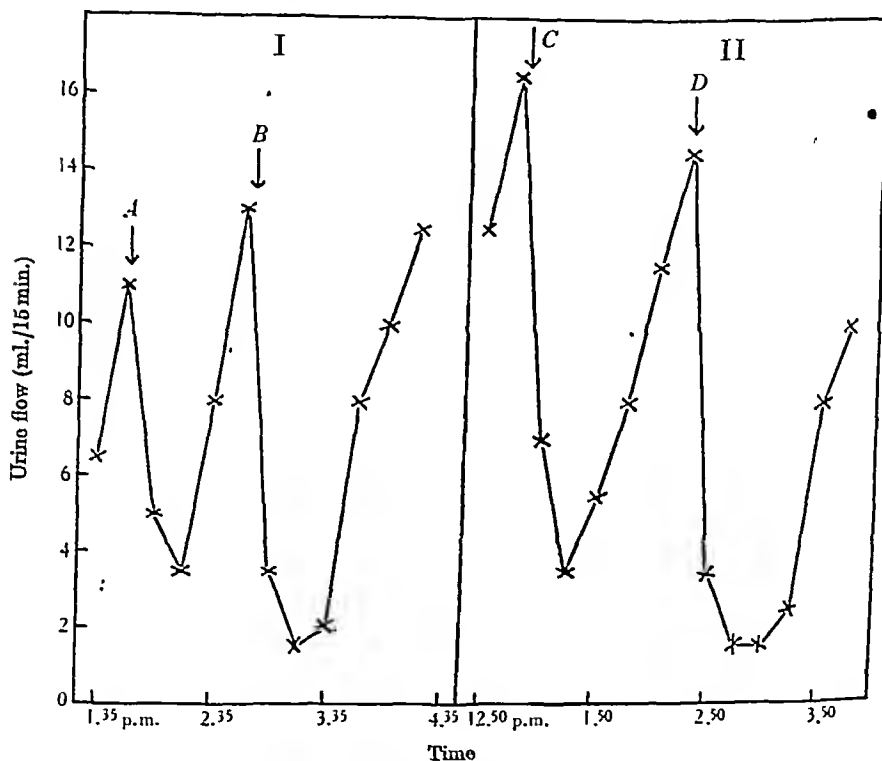


Fig. 1. Estimation of antidiuretic activity of grass-snake pituitary gland. Grass-snake no. 4, 50 g. ♂. I=rabbit no. 71. At 10.00 a.m. and at 12.10 p.m. 5% of body weight of water by stomach tube. A=1.0 mU. pitressin i.v. B=2.0 vol. % of grass-snake pituitary gland extract i.v. II=rabbit no. 89. At 10.04 a.m. and at 12.09 p.m. 5% of body weight of water by stomach tube. C=0.75 vol. % of grass-snake pituitary gland extract i.v. D=1.0 mU. pitressin i.v. The antidiuretic activity of the grass-snake pituitary extract was, therefore, equivalent to more than 50 mU. and to less than 130 mU. pitressin.

diuresis of a rabbit provided that care is taken not to excite the animal. To avoid excitement and struggling, it was found best to inject the rabbits without the help of a box or the hands of an assistant. Varying amounts of the extract of one reptile pituitary gland were matched with the response to a

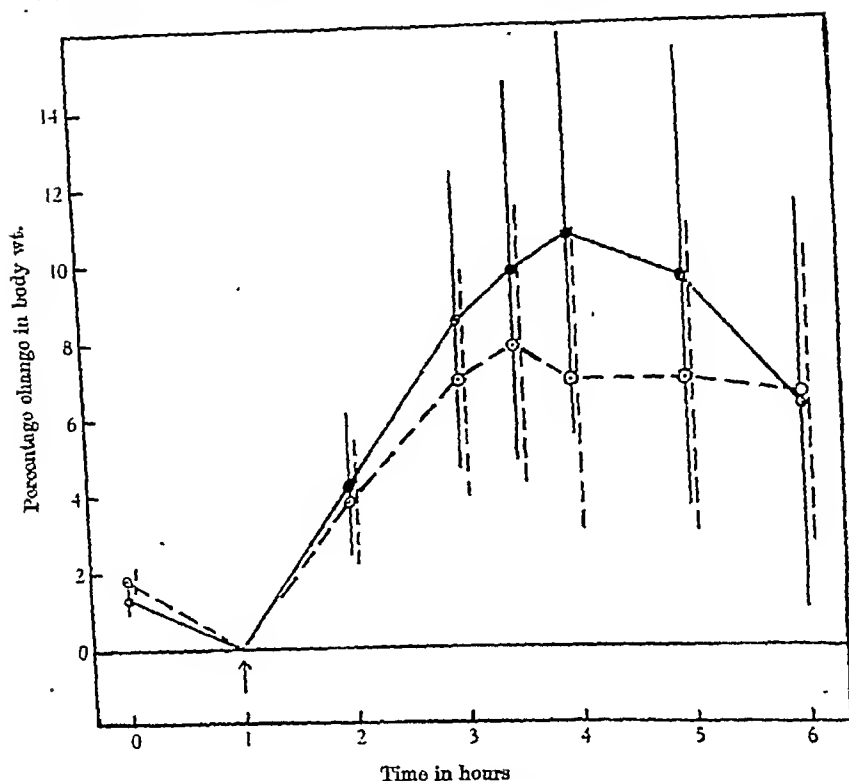


Fig. 2. Estimation of water-balance activity of grass-snake pituitary gland. ●—● mean percentage change in body weight of twenty frogs injected with 15 vol. % of an extract of one grass-snake pituitary gland each. ○----○ same frogs injected with 500 mU. of B.D.H. posterior pituitary extract each. The values for standard errors indicated by broken lines and belonging to the graph indicated by broken lines were obtained at the same relative times as those shown in full lines, but are placed alongside for technical reasons. Injections at the time marked by arrow.  $t$  (for maximum increases of weight) = 0.44,  $P < 0.7$ . It follows that the grass-snake pituitary glands contained about 3300 mU. of the water-balance principle.

TABLE 2. Water-balance activity and antidiuretic activity of pituitary glands of representatives of various classes of vertebrates

Class of vertebrate	Species of vertebrate used	Average amount of water-balance principle contained in one pituitary gland (in mU.). Figures in brackets = hormone content per 100 g. animal	Average amount of antidiuretic principle contained in one pituitary gland (in mU.). Figures in brackets = hormone content per 100 g. animal	Ratio of water-balance activity to antidiuretic activity per gland
Teleost fishes	Cod	8000 (—)	166 (—)	48.0/1.0
Amphibians	Frog	800 (4100)	3.5 (12)	228.0/1.0
Reptiles	Grass-snake	3300 (3600)	100 (95)	33.0/1.0
Birds	Pigeon	1500 (430)	31 (7)	48.0/1.0
Mammals	Rat	400 (240)	1075 (360)	0.4/1.0

TABLE 1. The antidiuretic activity of grass-snake pituitary glands. For description of method of estimation see 'Methods' and legend of Fig. 1

No.	Sex	Weight of animal in g.	Antidiuretic activity of pituitary gland in terms of mU. pitressin	Antidiuretic activity per 100 g. animal
1	♂	99	> 20 ~ 50	> 20 ~ 50
2	♂	82	~100 <200	~120 <240
3	♂	70	> 50 ~100	> 70 <140
4	♂	50	> 50 <130	>100 <260
5	♂	50	> 50 <100	>100 <200
6	♂	182	> 60 <100	> 35 < 50
7	♂	163	<200	<120
8	♂	121	> 50 <100	> 40 < 80
9	♂	175	~150 <200	~ 85 <115

*The (amphibian) water-balance activity of grass-snake pituitary glands.* Seven grass-snake pituitary glands were used for the estimations of water-balance activity. The extracts of several glands were pooled for any one experiment. Fig. 2 records one of these experiments. It will be seen that the water-balance activity of one snake pituitary gland equalled approximately that of 3300 mU. of a mammalian posterior pituitary extract. Other experiments gave essentially similar results. The average antidiuretic activity of grass-snake posterior pituitary glands, as established in the previous section, can be taken as roughly 100 mU. per gland. The ratio of water balance to antidiuretic activity is, therefore, approximately 33 to 1. This ratio is similar to that found for birds and fishes [Heller, 1941b], but of a different order of magnitude than that of mammals and amphibians (Table 2).

*Pressor assays of grass-snake pituitary gland extracts.* The number of experiments performed was not sufficient to permit a satisfactory assessment of the average pressor activity of grass-snake pituitary glands. However, the five experiments done did show the presence of considerable amounts of pressor activity (Fig. 3). Moreover, it was noticed that the apparent pressor activity of any one grass-snake pituitary gland was much in excess of the antidiuretic activity of the same extract (cp. e.g. Fig. 3 and Table 1, no. 8). A similar, though not as marked, discrepancy had previously been observed between the pressor and the antidiuretic potency of pigeon posterior pituitary lobe extracts [Heller, 1941a]. It is impossible to decide at present whether this discrepancy is due to a real difference between the concentration of the two activities in the extracts or whether it results from the combined errors of the methods of assay.

It will be observed that a not inconsiderable fall of blood pressure precedes the pressor effect of injections of snake pituitary extract (Fig. 3). This phenomenon occurred with all the extracts tested. It may be that this depressor effect is due to the presence of traces of a histamine-like substance. It is interesting to note that the depressor effect was only observed with grass-snake, and to a minor degree with frog pituitary extracts. It was not observed with

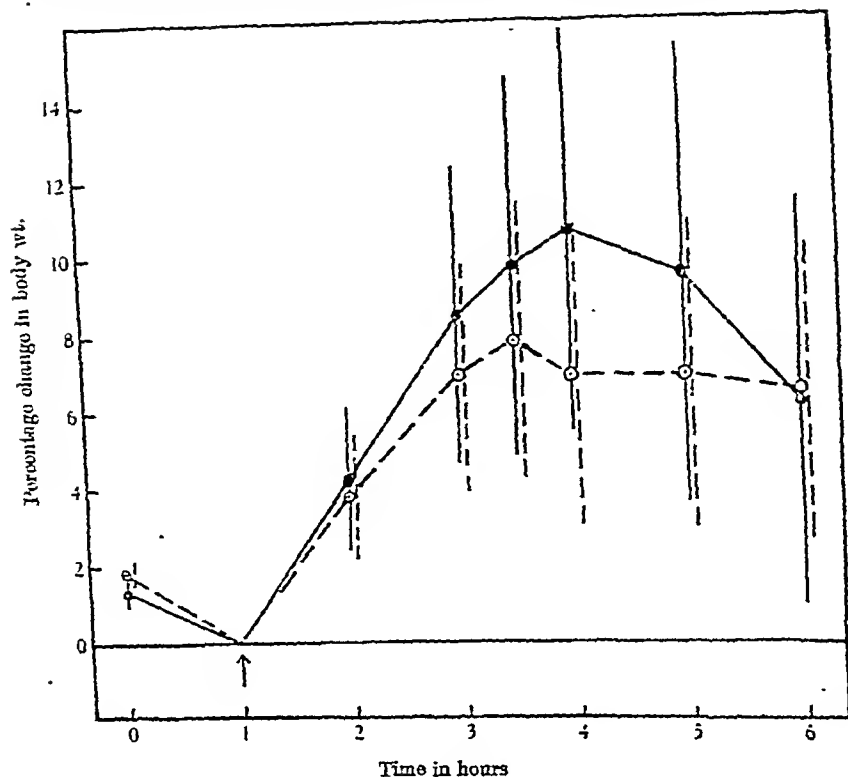


Fig. 2. Estimation of water-balance activity of grass-snake pituitary gland. ●—● mean percentage change in body weight of twenty frogs injected with 15 vol. % of an extract of one grass-snake pituitary gland each. ○---○ same frogs injected with 500 mU. of B.D.H. posterior pituitary extract each. The values for standard errors indicated by broken lines and belonging to the graph indicated by broken lines were obtained at the same relative times as those shown in full lines, but are placed alongside for technical reasons. Injections at the time marked by arrow.  $t$  (for maximum increases of weight) = 0.41,  $P < 0.7$ . It follows that the grass-snake pituitary glands contained about 3300 mU. of the water-balance principle.

TABLE 2. Water-balance activity and antidiuretic activity of pituitary glands of representatives of various classes of vertebrates

Class of vertebrate	Species of vertebrate used	Average amount of water-balance principle contained in one pituitary gland (in mU.). Figures in brackets = hormone content per 100 g. animal	Average amount of antidiuretic principle contained in one pituitary gland (in mU.). Figures in brackets = hormone content per 100 g. animal	Ratio of water-balance activity to antidiuretic activity per gland
Teleost fishes	Cod	8000 (—)	166 (—)	48.0/1.0
Amphibians	Frog	800 (4100)	3.5 (12)	228.0/1.0
Reptiles	Grass-snake	3300 (3600)	100 (95)	33.0/1.0
Birds	Pigeon	1500 (430)	31 (7)	48.0/1.0
Mammals	Rat	400 (240)	1075 (360)	0.4/1.0

extracts of mammalian and avian pituitary glands. All extracts were prepared immediately after the death of the animal. The occurrence of the histamine-like effect is, therefore, not due to post-mortem decomposition of the glandular material. It is quite conceivable, however, that the method of extraction removes a histamine-like substance in one instance, e.g. from snake pituitary glands and does not remove it in another, e.g. from mammalian glands. Grant & Jones's [1929] report on the presence of a vaso-dilator substance in the skin of frogs with similar but not identical properties to the histamine-like substance in mammalian skin, may be recalled in this connexion.

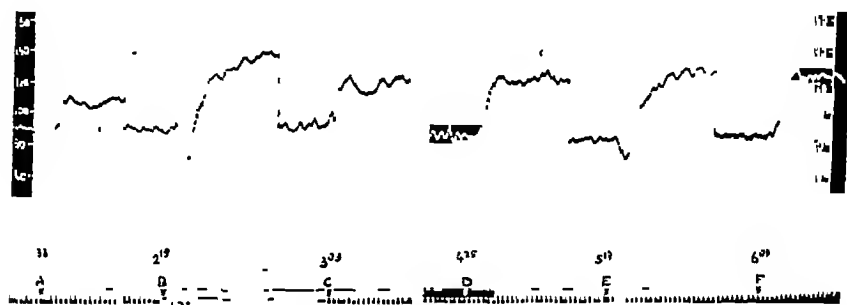


Fig. 3. Pressor activity of grass-snake pituitary gland extract. Spinal cat. Extract of grass-snake pituitary gland no. 8. *B* and *E*=intravenous injection of 25 vol. % of grass-snake pituitary extract. *A*=intravenous injection of 30 mU. pitressin. *C*=50 mU. pitressin i.v. *D*=70 mU. pitressin i.v. *F*=90 mU. pitressin i.v. All injections made up to 1 c.c. The vasopressor activity of the whole gland equals approximately 320 mU. pitressin.

Mixtures of mammalian posterior pituitary extracts with amounts of histamine-acid phosphate having a proportionately equal depressor effect to the vaso-dilator impurities in snake pituitary extracts, had much the same pressor effect as the same amounts of pure posterior lobe extract without the histamine. It is, therefore, unlikely that the traces of depressor substance found in grass-snake pituitary extracts influenced the pressor assay to a significant degree.

#### DISCUSSION

It is clear from these experiments that the posterior pituitary glands of grass-snakes contain considerable amounts of active principles. This result agrees with the anatomy of the snake pituitary gland, the pars nervosa of which is a well-developed structure. Teleologically it would have been surprising if little or no activity had been found in the posterior pituitary gland of a snake, i.e. of a member of a vertebrate order which counts some of the most aridly living animals among its numbers, an order, therefore, in which a highly developed mechanism for the conservation of water would be expected.

The results of the quantitative determinations of the water-balance activity and of the antidiuretic activity of grass-snake pituitary glands will be found in Table 2, which includes the results obtained for pituitary gland extracts of representatives of the phylogenically adjacent classes of vertebrates [Heller, 1941*a*, 1941*b*]. However, no attempt will be made to draw conclusions from the quantitative data obtained for the different classes, as there are several considerations which render the significance of these values difficult to interpret. Some such factors as the errors of assay and the influence of captivity, involving possibly changes in the water uptake of the experimental animals [Geiling, 1940], may be of minor importance if the large differences found between the hormone content of glands of different classes are considered. However, two other considerations may be of more serious significance: (1) Only one or in some cases two species of any particular class of vertebrates have been investigated. It must be admitted that, so far, the differences between the hormone content of glands of different species have been found to be significantly smaller than the differences between the hormone content of glands of animals belonging to different classes of vertebrates. But data on more species, including if possible related species with a widely different habitat, are desirable. (2) It will be noted that the quantitative determinations of the posterior pituitary activities of the frog and of the rat suggest a relatively much higher concentration of the water-balance principle and the antidiuretic principle respectively for these than for the representatives of the other classes of vertebrates. On the other hand, it is known that amphibians and mammals, respectively, respond to these principles in a specific manner (Table 3).

TABLE 3. Effect of posterior pituitary extract on the water metabolism of different classes of vertebrates

	Extrarenal ('water-balance') effect	'Glomerular' antidiuresis	'Tubular' antidiuresis
Amphibians	Pronounced	Doubtful	None
Reptiles	Doubtful	Pronounced	None
Birds	Not investigated	Feeble	Feeble
Mammals	None*	None	Pronounced

\* Boyd & Garand [1942] showed recently that pitocin, i.e. the posterior pituitary fraction containing the bulk of the amphibian water balance activity, is not more effective than pitressin but rather less so in retaining body water in a mammal, the albino rat. These results differ from those obtained in corresponding experiments on frogs [Heller, 1930; Boyd & Brown, 1933] and indicate that posterior pituitary extract does not have the same effect upon mammalian water balance as upon amphibian water balance.

It would, therefore, be tempting to assume a correlation between a class specific receptor apparatus and the quantity of the corresponding hormone produced by the gland. However, the apparent preponderance of a pituitary principle in the glands of a class of animal on which that principle exerts a specific action is open to another interpretation. It has been shown in the case of anterior pituitary hormones and of the gonadotrophic hormone, in particular,

that the effectiveness of the hormone in a foreign species tends to vary directly with the phylogenetic proximity of the donor and the recipient species [Creaser & Gorbman, 1939]. In some instances the loss of effectiveness proved to be so great as to lead to an apparent refractoriness by the recipient species to rather large doses of gonadotrophic material. It becomes clear, therefore, that if there is an appreciable variation of responsiveness of a given test animal to hormones obtained from different species or classes, then 'units' as determined by biological tests do not represent equivalent absolute units of hormones, but only the amount of hormone required to produce a given effect on the particular animal species or class under consideration. Though the possibility of species or class specificity of posterior pituitary principles seems so far not to have been suggested, it will be realized that the data indicating a preponderance of the antidiuretic principle in mammalian glands and of the water-balance principle in amphibian glands have been obtained in experiments with material from mammals and frogs respectively. The apparent high concentration of the antidiuretic principle in mammals and of the water-balance principle in the frog may, therefore, be an indication of the class specificity of these principles.

The difficulty of visualizing the mechanism of a posterior pituitary effect on the water metabolism of reptiles has been mentioned, and it was pointed out that, as far as we know, reptiles lack the effector mechanisms of the phyletically adjacent classes. That is to say, reptiles show neither the rapid water movement through the skin as, e.g. frogs, nor are their renal tubules specially adapted for water reabsorption beyond the iso-osmotic level. Assuming that water conservation is secured by the secretion of the posterior pituitary lobe in reptiles as in other classes of vertebrates, on what mechanism could it be based?

It should be remembered that, taking the vertebrate phylum as a whole, we know of three actions of posterior pituitary extracts which may influence the body water of animals: (1) The increase of tubular water reabsorption as present in mammals and birds. (2) The increase of the permeability of the skin to water as in amphibians. (3) The constrictor action on blood vessels and on the glomerular capillaries in particular. The third mechanism has so far not been drawn into the circle of physiological possibilities, but it is clear that an antidiuresis caused by a decrease of the glomerular filtrate would tend to conserve water. Such a 'glomerular antidiuresis' induced by posterior pituitary extracts has been occasionally observed in amphibians and in mammals [Adolph, 1936; Iversen & Bjerring, 1934]. However, very large doses had to be given to produce it. The question arises whether the posterior pituitary vasopressor principle acts on reptilian kidneys in a similar manner, and whether the reptile glomerulus is more sensitive to its action than the glomerulus of other vertebrate classes.



Burgess, Harvey & Marshall [1933], who investigated the action of mammalian posterior pituitary extracts on the urinary secretion of various classes of vertebrates, found that even very large doses of pitressin (1000 mU. per kg.) did not produce an inhibition of urine flow in the frog (*R. catesbiana*). However, the antidiuretic action of 100 mU. per kg. in a reptile (the alligator) was very pronounced. Marshall and his co-workers investigated this antidiuretic action in the alligator further by measuring the xylose clearance, and found that 'even very small doses of pitressin decreased the glomerular filtrate almost proportionately to the urine flow'. This finding is in contrast to the antidiuretic action of posterior pituitary extracts in mammals which has been repeatedly shown to occur without a significant decrease in glomerular filtration.

With all reservation as to future findings in other species of reptiles it can be pointed out, therefore, that the experimental evidence in the one reptile so far investigated does not contradict the hypothesis that water conservation in this vertebrate class may be effected by a decrease in the volume of the glomerular filtrate.

A serious objection may be raised against regarding such a mechanism as of physiological importance. Decreasing the glomerular filtrate means not only a decrease of water lost in the urine, but also a decrease of the glomerular excretion of metabolic waste products—clearly an unsatisfactory state if constriction of the glomerular capillaries lasts for any length of time. However, it should be remembered that, in contrast to mammals, the metabolic waste products and especially uric acid of reptiles are eliminated not only by glomerular filtration but to a much larger extent by tubular secretion [Marshall, 1932], and that tubular secretion has been shown to be highly independent of glomerular activity (e.g. by Pitts [1938] for the avian kidney). Thus tubular secretion may be of greater importance for a reptile in which a 'glomerular antidiuresis' is a physiological phenomenon than for a mammal in which conservation of water can be achieved without interference with the glomerular blood flow.

Accepting the possibility of a glomerular action of the posterior pituitary secretion in the reptile, how would it fit into a scheme of the known effects of posterior pituitary extracts on the water metabolism of the various classes of vertebrates? It will be seen (Table 3) that the data on reptiles agree with the findings on the other classes in that the prevalence of a class specific effector mechanism is equally indicated. Birds form an apparent exception, as there appear to be two important mechanisms of posterior pituitary action. This exception can possibly be explained by the fact that the kidney of birds consists anatomically of a mixture of mammalian-like and of reptilian-like nephra.

The difficulty of drawing generalized conclusions from the scattered and

incomplete evidence will be appreciated. However, three points worth mentioning are beginning to emerge.

1. The findings mentioned in this and in the other papers of this series [Heller, 1941a, 1941b] suggest that all the three posterior pituitary activities possibly concerned with the regulation of the metabolism of water are present in all classes of vertebrates.

2. The effect of one posterior pituitary activity in preference to the others and dependent on an effector mechanism specific for the class appears to prevail in each class of vertebrates.

3. The comparative physiology of the posterior pituitary action will probably be best understood by the application of a teleological concept, namely that of conservation of water.

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# THE EFFECT OF GRADED DOSES OF VITAMIN C UPON THE REGENERATION OF BONE IN GUINEA-PIGS ON A SCORBUTIC DIET

By GEOFFREY BOURNE,<sup>1</sup> *From the University Laboratory of  
Physiology, Oxford*

(Received 25 May 1942)

Both experimental and clinical observations [Höjer, 1923; Aschoff & Koch, 1919] have indicated that in scurvy it was the bony tissue which showed the most constant and characteristic lesions. Typical changes were the loosening of the attachments of the periosteum to the bone, the appearance of subperiosteal haemorrhages which often extended considerable distances along the shaft, and a thinning of the cortices of the long bones and of the bony trabeculae. In addition, the marrow was found to lose its red colour and to become pale and gelatinous in appearance.

The earliest worker to investigate experimentally the effect of scurvy on the regeneration of bone was Shinya [1922], who showed that a graft of bone from a scorbutic to a normal guinea-pig would not 'take' and that conversely the transplantation of a sound bone into a scorbutic animal was ineffective; although the early death of the animals from scurvy made prolonged investigation of the latter experiment impossible. Subsequent investigators found in scorbutic guinea-pigs delay in the formation of new bone in fracture calluses [Ferraris & Lewi, 1923; Israel & Frankel, 1926; Hanke, 1935; Hertz, 1936; Lexer, 1939] and in saw cuts in the cranial bones [Watanabe, 1924; Schilozew, 1928].

Halász & Marx [1932] have demonstrated that if vitamin C is given to guinea-pigs in amounts exceeding the normal requirement there is no acceleration of callus formation. On the other hand, Hanke [1935] and Giangrasso [1939] claimed that in rabbits, extra vitamin C did accelerate the healing of fractures. Bourne [1942], using what was probably a more accurate technique, was unable to show that extra vitamin C had any effect in accelerating the regeneration of bone in rats (which like rabbits synthesize their own vitamin C and can be presumed therefore to be saturated with it).

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Although some of the workers mentioned have used mild or severe scurvy in their experiments none appear to have made any attempt to find the critical value for the amount of vitamin C required to promote optimum regeneration of bone. This is the object of the present work.

### METHODS

Twenty-eight young male guinea-pigs of approximately the same age and weight were placed on a scorbutic diet. The diet was composed of a daily mash of crushed oats, wholemeal flour, cod-liver oil and water, and of a hard cake which was provided in excess. The cake contained 'weatings', ground wheat, ground oats, ground barley, ground maize, meat and bone meal, dried skim milk, salt, dried yeast, fish meal and cod-liver oil. The animals were given varied doses of vitamin C by subcutaneous injection each day of the amounts shown below:

- 5 received 0.25 mg.
- 4 received 0.50 mg.
- 5 received 1.00 mg.
- 5 received 2.00 mg.
- 4 received 4.00 mg.

At the end of 1 week on this diet a hole, 1 mm. in diameter, was bored aseptically in both femora of each guinea-pig (for details see Bourne, 1942). Standard holes of this size provided exactly comparable areas for regeneration in each animal. After 7 days the animals were killed and the femora fixed, decalcified, sectioned and stained with haematoxylin and van Gieson.

### RESULTS

#### *Animals receiving no vitamin C*

There is considerable variation in the histological picture between the femora of various animals in this group and in some cases between the right and left femora of the one animal. This latter finding indicates that local factors may play as important a part, under certain circumstances, as systemic factors. One of the first normal responses to bone injury is usually a multiplication of cells in what is called the 'cambial layer' of the periosteum, that is, the inner layer of the periosteum near the bone. Even this reaction was absent in some of the femora of this group. Occurring more or less simultaneously with the cambial reaction is the aggregation of fibroblasts in the clot and the beginnings of organization, i.e. the production of fibres, presumably by the activity of the fibroblasts, which, according to Danielli [1942], may spin such fibres from a layer of protein adsorbed on to their surfaces. Fibroblasts are present in the clot in most of the femora but in some they appear to have produced no fibres. The clot was brittle and in some sections had crumbled, presumably because

of the treatment it had received in sectioning process. In most of the femora, however, the clot was filled with colourless pre-collagen fibres. In some, a few of these fibres stained a faint pink with van Gieson, indicating that they had changed to mature collagen. In others there were aggregations of structureless material which stained faintly or not at all and probably represent swollen, oedematous masses of pre-collagen. In other femora there was developed in the fibrous clot a number of small islands of van Gieson staining material. In about half the femora examined there was no periosteal reaction and in none was there any reaction on the part of the endosteum. This is of

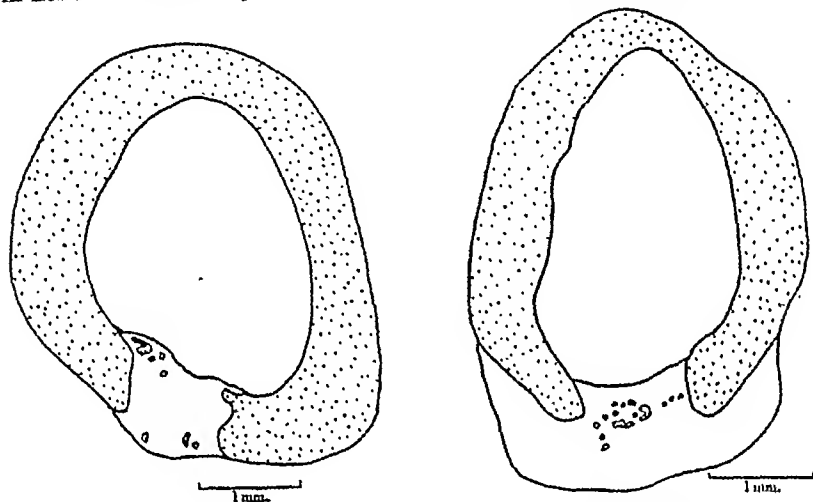


Fig. 1.

Fig. 2.

Fig. 1. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with no supplement of vitamin C.

Fig. 2. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet supplemented with 0.25 mg. vitamin C per day.

For lettering see Fig. 6.

interest because in those animals which have adequate supplements of vitamin C the endosteum produces a number of trabeculae which link up with those forming in the inner part of the hole. In some femora small chips of what appeared to be dead bone, and which had lodged in the hole as a result of the operation, showed no sign of resorption. All the cells present in the holes appeared to be fibroblasts, there was no sign of osteoblasts.

#### *Animals receiving 0.25 mg. vitamin C*

The most noteworthy effect of the vitamin C in this series was that in every femur except one there was a distinct reaction on the part of the cambial layer of the periosteum, but there was no formation of trabeculae between it and the uninjured bone as occurs in normal periosteal reactions. None of the femora showed any endosteal reaction. There was, as in the previous group,

one femur in which there was no reaction at all to the injury. The marrow had oozed through the hole and there was no accumulation of fibroblasts and fibres in the region of the hole.

*Animals receiving 0.5 mg. vitamin C*

In all the femora examined in this group there was a distinct reaction on the part of the cambial layer. Although there was multiplication of the cells in this region there was no formation of trabeculae between the bone and the periosteum. Four out of seven femora examined showed a considerable reaction on the part of the periosteum, which was particularly intense near the hole.

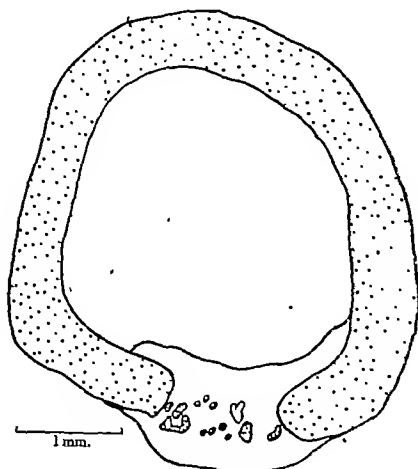


Fig. 3.

Fig. 3. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 0.5 mg. vitamin C per day.

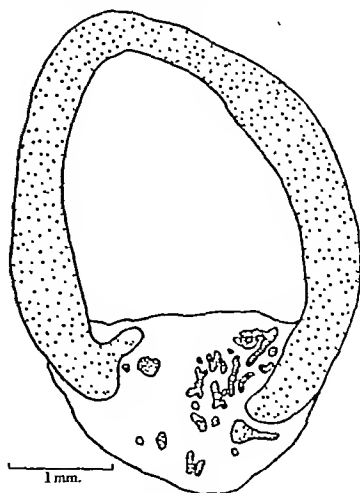


Fig. 4.

Fig. 4. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 1.0 mg. vitamin C per day.

For lettering see Fig. 6.

In four femora there were the beginnings of an endosteal reaction, i.e. a few small trabeculae which appeared to be formed from the endosteum near the margin of the hole. Most femora contained a number of fibres in the hole, but while in one femur of one animal there was a mass of dense fibres, in the other femur the hole contained a number of trabeculae. A number of osteoblast-like cells were present in the hole in most femora and some capillaries were present.

*Animals receiving 1 mg. vitamin C*

In all the eight femora examined in this group there was a considerable reaction on the part of the periosteum and the endosteum. The former showed not only cellular multiplication of the cambial layer but in most cases the



formation of bony trabeculae in this layer between the periosteum and the surface of the bone, in other words the osteogenetic power of the periosteum had been retarded up to this point. The endosteum had produced bony trabeculae in most of the femora in the immediate precincts of the hole. The hole itself was in all cases densely packed with fibres which did not stain with van Gieson. In a few of the femora some of these fibres, although not arranged in trabeculae, stained with varying intensity with van Gieson. There were many capillaries in the hole and numerous osteoblasts were present, particularly in regions where trabeculae were forming.

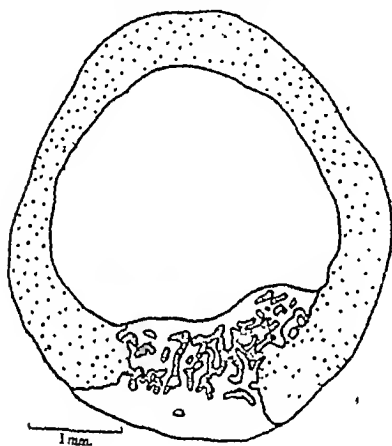


Fig. 5.

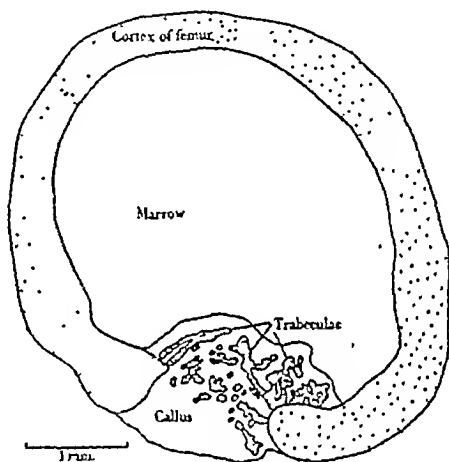


Fig. 6.

Fig. 5. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 2.0 mg. vitamin C per day.

Fig. 6. Projection drawing of section through the hole in the femur of a guinea-pig on a scorbutic diet with 4.0 mg. vitamin C per day.

#### *Animals receiving 2 mg. vitamin C*

In most of the femora of this group the inner part of the hole (near the endosteum) was filled with bony trabeculae which linked with those formed by the endosteum. The outer part of the hole was packed with fibres many of which stained with van Gieson. The periosteum in all members of this group appeared to have attained its full osteogenetic power and to have produced large numbers of thick trabeculae in association with the uninjured bone, but it had not formed many trabeculae in the outer part of the hole. Possibly this stage does not appear in the guinea-pig until later in the healing process. In a rat in most cases such a hole is completely filled with trabeculae at the end of a week. There were many osteoblasts and capillary blood vessels present in the holes of all animals.

*Animals receiving 4 mg. vitamin C*

In the femora of this group too there was considerable periosteal and endosteal reaction with the formation of bony trabeculae by both. Most of the holes contained numerous fibres, but generally speaking there appeared rather less van Gieson staining material than in the preceding group. This fact is illustrated in figures given later. There were numerous osteoblasts and capillary blood vessels present in the tissue filling the holes in all animals.

*Summary of changes*

Dose of vitamin C mg.	Periosteal reaction	Endosteal reaction	Fibres in hole	Trabeculae
None	Negative	Negative	A few	None
0.25	Slight multiplication of cells in cambial layer	Negative	Fairly numerous	None
0.50	Considerable multiplication of cells in the cambial layer	Slight reaction. Beginnings of formation of trabeculae	Numerous	None
1.00	Cambial layer forming trabeculae	Small number of trabeculae forming	Very large numbers	A few
2.00	Very large reaction. Enormous multiplication of cambial layer cells. Many trabeculae formed	Extensive formation of trabeculae	Very large numbers	Numerous, particularly in inner part of hole
4.00	Ditto	Ditto	Ditto	Ditto

An attempt was also made to obtain figures which would indicate the degree to which the formation of new bone has occurred in various groups of animals. A technique for this purpose has been described by Bourne [1942]. Briefly it is as follows.

The trabeculae in five random sections taken through the region of the middle of the hole in each femur were projected on to paper and drawn. The weight of a standard rectangle of paper which fitted into the hole was obtained and the trabeculae contained in this rectangle were drawn and subsequently cut out and weighed. The division of the second weight by the first gave a figure called the 'trabecular index'. Since some of the indices obtained in this work were so small it was found necessary for convenience to multiply all indices by 100.

The results obtained by the application of this technique were as follows:

				Trabecular index times 100
5 animals on a scorbutic diet.	No supplement of vitamin C			7.73 $\pm$ 2.25
5 " " "	Supplement 0.25 mg. vitamin C			6.70 $\pm$ 2.30
4 " " "	" 0.50 mg. "			9.74 $\pm$ 2.48
5 " " "	" 1.00 mg. "			19.41 $\pm$ 3.53
5 " " "	" 2.00 mg. "			23.73 $\pm$ 6.75
4 " " "	" 4.00 mg. "			18.09 $\pm$ 4.48

The apparent decrease in the amount of trabeculae formed in the group receiving 0.25 mg. vitamin C compared with that of the animals receiving no vitamin C supplement, is probably spurious. In these two groups, and also in the next group (that receiving 0.50 mg. of vitamin C per animal per day), there was no formation of true trabeculae. Small spots of van Gieson staining material were found and occasionally relatively large areas which appeared to have been formed by the clumping together and fusing of pre-collagen fibres were seen. These masses stained very faintly, and it was difficult to decide whether to include them as trabeculae or not. However, they were included and since there were rather more of them in the completely scorbutic group than in the group receiving 0.25 mg. vitamin C, their inclusion as trabeculae weighted the results towards the former group.

There was a slight increase in the amount of van Gieson staining material present in the next group (0.25 mg. vitamin C).

In the 1 mg. group there was a considerable increase in the van Gieson staining material, and in this group for the first time the isolated islands of van Gieson staining material linked up to form trabeculae. In the 2 mg. group this process was carried a stage further. The drop in the amount of van Gieson staining material in the 4 mg. group may be due to the retarding effect of the higher dose of vitamin C, but it seems more likely to be due to experimental error. In fact it is very difficult satisfactorily to apply the technique of estimation of trabeculae to this work owing to the difficulty in the scorbutic and partially scorbutic animals of deciding what material to include as trabeculae and what not to include; under the circumstances the application of Fisher's 't' test for significance to the figures would be of little value.

#### DISCUSSION

These results show that without adequate vitamin C there is practically a complete inhibition of the reparative processes in a damaged bone. As the dose of vitamin C increases, one reparative function after another comes to life, until, with a daily supplement of 2 mg., regeneration appears to be taking a normal course. The critical dose lies somewhere between 1 and 0.5 mg. per day as can be seen from the list of trabecular indices. There is still further improvement in the histological picture when 2 mg. vitamin C are given per day but no apparent further improvement at the 4 mg. level.

The effects of complete deficiency of vitamin C obtained in this work are similar to those obtained by other workers [e.g. Wolbach & Howe, 1926; Hertz, 1936]. Hertz points out that in a completely scorbutic animal, following bone injury, there is no periosteal hyperaemia, and that in general it is difficult for a scorbutic animal to produce an inflammatory reaction in response to injury, and that, in the absence of such a reaction, the normal processes of healing cannot be introduced. The enlargement of the cambial

layer of the periosteum, Hertz believes to be an essential stage of this inflammatory reaction.

Hertz [1936], like Watanabe [1924], noticed a delayed absorption of the haematoma at the site of the fracture in scorbutic animals. The same phenomenon has been observed in this work. In the animals receiving no vitamin C and in those receiving 0.25 mg. of the vitamin, at the end of the experimental period (7 days after injury) the whole of the connective tissue supporting the muscles associated with the femur was permeated with blood which had not been absorbed. The impression given was that the blood had not clotted very readily and that it had continued to ooze from the hole for some time after the operation. Finally, it extended through practically the whole of the soft tissues of the upper part of the leg. The connective tissue was (as has been found by other workers) obviously oedematous and had a soft jelly-like appearance. The animals receiving 0.5 mg. of vitamin C showed a small haematoma at the site of injury, but in the 1.00 mg. group and the 2.0 and the 4.0 mg. group it had disappeared by the time the animals were killed.

This work confirms the results of other workers that there is a delay in the development of collagen fibres and delay in their maturation [e.g. Hunt, 1941] in scurvy.

In view of the present war situation it would be of interest to attempt to interpret the results with guinea-pigs in terms of human requirements. This, however, is no easy task.

Göthlin [1934] has stated that the stage of scurvy in guinea-pigs which is recognizable only by means of microscopic alterations in the teeth is equivalent to that prescorbutic stage in man which is indicated by fragility of the capillaries. He found that such teeth changes could be prevented in the guinea-pig by a minimum dose of 1.33 mg. a day of vitamin C and that 19-27 mg. a day were required to prevent capillary changes in man. Therefore the factor for converting guinea-pig doses of vitamin C to human doses should be approximately 17, and anything less than 17 mg. of vitamin C a day for a human being with a fractured bone would be likely to retard seriously the healing of the fracture and 34 mg. a day would be required to secure the maximum formation of callus.

On the other hand, Kellie & Zilva [1939] state that the daily dose necessary to maintain the condition of saturation in a human being is 30-40 mg., and that a guinea-pig requires for the same purposes about 20 mg. [Zilva, 1936]. These results suggest that a human being needs only twice as much vitamin C as a guinea-pig, and that therefore 4 mg. of the vitamin per day in a human being with an injured bone would be sufficient to induce regenerative changes equivalent to those obtained in the guinea-pig with 2 mg.

But a guinea-pig requires 0.5-1 mg. of vitamin C a day to protect it from macroscopic signs of scurvy, and Fox, Dangerfield & (

that a daily intake of 12-25 mg. of vitamin C resulted in the appearance of scurvy in twelve out of 950 mine labourers studied. Apparently then an intake of this order is approaching the scorbutic danger level. The vitamin C demands of mine labourers may be greater than those of persons lying in bed in hospital with a fracture; but even so it seems that anything less than 10 mg. a day in such a patient would be undesirable. If we accept 10 mg. of vitamin C as the possible minimum amount required to prevent macroscopic scurvy in such patients, then we see that it is necessary to multiply a guinea-pig dose by 15-20 in order to obtain the human equivalent. Therefore to secure the same degree of bone healing present in a guinea-pig with 2 mg. of vitamin C a day, human beings should receive about 40 mg., and anything less than 20 mg. would seriously retard the healing process. Fox *et al.* [1940], however, found no evidence that fractures or wounds healed more rapidly in mine labourers on 40 mg. of vitamin C than on those receiving 12-25 mg. per day. But they admit that comparison was difficult owing to the difference in situation and severity of the lesions.

There are many other complications, for example Crandon [see Crandon, Lund & Dill, 1940] found that it took him 169 days to develop macroscopic scurvy on a vitamin C-free diet, Wolbach & Howe [1926] found that when the bones of scorbutic guinea-pigs were injured there was a temporary healing process initiated in the incisor teeth, and Lauber, Nafziger & Bersin [1937] found that a complete fracture of the humerus in rabbits caused an increase in the excretion of vitamin C.

Nevertheless, if we assume that 40 mg. vitamin C a day is needed to secure maximum regeneration of injured bone then the results are in keeping with recent observations on the vitamin C requirement of man [see Smith, 1938; Harris, 1941]. In any case it is desirable that patients with fractured bones should be given at least 40 mg. vitamin C a day as long as there is the slightest doubt about the efficacy of smaller doses.

#### SUMMARY

1. The effect of the injection of graded doses of vitamin C on the regeneration of bone in guinea-pigs has been investigated by measuring the amount of trabeculae formed in a hole bored in the femur at the end of one week.
2. It has been found that guinea-pigs require 2 mg. of vitamin C a day by injection to secure adequate regeneration of bone and that less than 1 mg. seriously retards regeneration.
3. It is suggested that the corresponding doses to produce the same results in human beings may be 40 mg. and 20 mg. vitamin C.
4. Pure synthetic vitamin C is alone able to promote regeneration of bone in scorbutic guinea-pigs.

I am indebted to Roche Products for a supply of vitamin C for these experiments.

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THE ACTIVITY OF *l*(-)-DOPA DECARBOXYLASE

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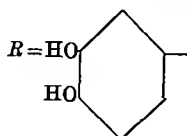
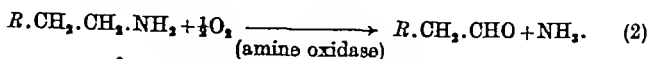
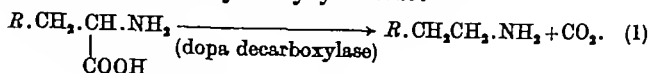
The breakdown of amino acids in the mammalian body is generally initiated by the oxidative removal of the amino group. If amines such as adrenaline or histamine are formed from amino acids, it would appear probable that in these instances at least the amino group is retained. Decarboxylation is easily demonstrated in the metabolism of amino acids in bacteria. In mammalian tissue, decarboxylases for various amino acids have been claimed to occur, but definite proof has been given for one amino acid only, *l*(-)-dopa [*l*(-)-(3:4)-dihydroxyphenylalanine]; the amine formed in the reaction is hydroxytyramine [ $\beta$ -(3:4)-dihydroxyphenylethylamine].<sup>1</sup>

The present paper deals with the question whether the decarboxylase is specific for *l*(-)-dopa, and whether tissue extracts contain other amino acid decarboxylases of a comparable activity. The close structural relationship between hydroxytyramine and adrenaline was the reason for including a number of *N*-methylated amino acids as substrates in our experiments. In addition, some data are given on the properties of *l*(-)-dopa decarboxylase and its distribution in different species, including Man. Since no data are available on amine oxidase in human tissue, we have taken the opportunity of examining the human extracts for the presence of this enzyme.

*l*(-)-Dopa was first prepared from *Vicia faba* [Guggenheim, 1913], and it was suggested that it might be the precursor of the melanotic pigment in mammalian skin [Bloch, 1916]. Hydroxytyramine, prepared by Mannich & Jacobsohn [1910] and by Barger & Ewins [1910], has a pressor action on the arterial blood pressure of the cat about 1/35 of that of adrenaline [Barger & Dale, 1910]. The effect is sensitized by cocaine and reversed by ergotoxine [Tainter, 1930]. The amine occurs in broom [*Cytisus scoparius* Link], where it is believed to be responsible for the autumnal blackening of the pods [Schmalfuss & Heider, 1931]. It can replace *l*(-)-dopa in the 'dopa reaction' of the human skin [Mulzer & Schmalfuss, 1931].

<sup>1</sup> Throughout this paper the shorter names *l*(-)-dopa and hydroxytyramine will be used.

There are two enzymic systems, one of which catalyses the formation, the other the decomposition, of hydroxytyramine:



The amine oxidase present in extracts of guinea-pig's kidney and liver oxidized hydroxytyramine more quickly than any other of its known substrates, e.g. adrenaline or tyramine (equation (2)) [Blaschko, Richter & Schlossmann, 1937]. In 1938 Holtz, Heise & Lüdtke discovered in the kidney of the guinea-pig and some other mammals an enzyme which decarboxylated *l*(-)-dopa to hydroxytyramine and  $\text{CO}_2$  (equation (1)). They identified the hydroxytyramine chemically and measured the formation of  $\text{CO}_2$ . Owing to the presence in the extracts of both amine oxidase and *l*(-)-dopa decarboxylase satisfactory yields of hydroxytyramine were obtained only when the oxidation of the amine formed was prevented either by removal of oxygen or by addition of an inhibitor of amine oxidase, e.g. octyl alcohol. The decarboxylase also occurs in the guinea-pig's liver [Blaschko, 1939; Holtz, 1939] and intestine [Holtz, Credner & Reinhold, 1939], in the liver and kidney of rabbits, sheep, goats, pigs and hens, but not in those of rats, mice and cattle [Holtz, Credner & Walter, 1939-40].

#### MATERIAL AND METHODS

**Enzyme preparations.** The organs used for the preparation of extracts were ground in a mortar with sand; equal amounts of *M*/15 sodium phosphate buffer pH 7.4 were added and the extracts were centrifuged for 5 min. The opaque supernatant fluid was used. The tissue extracts have, except where otherwise stated, been obtained from the guinea-pig's liver

The human tissue consisted of pieces of three kidneys obtained from operations performed under spinal anaesthesia. In two cases (*A* and *B*) the diagnosis was stones in the pelvis and pyonephros, the third was a case of tuberculosis of the kidney (*C*). From *A* only 0.55 g. tissue were obtained and the procedure for preparing the extract had to be modified. The tissue was brought to the laboratory from the site of operation in a thermos flask at  $-10^\circ \text{C}$ . 1 hr. after the operation; it was weighed, ground without sand in a special mincer for small amounts of tissue and taken up in 2.0 c.c. *M*/15 phosphate buffer. The extract was not centrifuged. The actual experiment was carried out 6 hr. later, during which time the material was kept at  $0^\circ \text{C}$ . The kidney from case *B* had a cortex slightly reduced in thickness, otherwise the tissue was macroscopically



and microscopically normal. The kidney of case *C* showed a few circumscribed tubercles in one pyramid, but the tissue from the other pole, which was used, was not affected and, apart from a few cylinders, had a normal microscopical picture. The preparation of the extracts from the kidneys *B* and *C* was made immediately after the removal of the kidneys and the extracts were tested without delay. Since the material was plentiful, the extracts were prepared in the same way as those from the animal tissues. I should like to thank Mr Harold Dodd and Dr G. Warnock for the material used in these experiments.

*Manometric technique.* The enzymic activity was determined manometrically by measuring the  $\text{CO}_2$  formed in the reaction. The extracts had a relatively high retention of  $\text{CO}_2$ , only a part of which appeared in the gas phase in the course of the reaction. The  $\text{CO}_2$  retained was, therefore, determined at the end of each experiment by driving it out with sulphuric acid. Conical manometer flasks of the Warburg-Barcroft type and simple manometers were used. The flasks had one side bulb and an inner ('potash') cylinder. The extracts were in the main flask and the experiment was started by tipping in the substrate solution from the side bulb. At the end of the experiment 0.3 c.c.  $N/1 \text{ H}_2\text{SO}_4$  were spilled from the inner tube into the main flask. In the experiments with mammalian tissue extracts the temperature was  $37.5^\circ \text{C}$ . In those with extracts from frog's tissue it was  $18.5$ – $20.5^\circ \text{C}$ . In most experiments the total volume in which the reaction took place was 2 c.c.: 1.6 c.c. extract in the main flask, if necessary, suitably diluted with phosphate buffer, and 0.4 c.c.  $M/100$  amino acid in the side bulb, making the initial substrate concentration  $M/500$ . In addition there was 0.3 c.c.  $N/1 \text{ H}_2\text{SO}_4$  in the inner tube. Unless otherwise stated the experiments were carried out in nitrogen.

*Substrates.*  $l(-)$ -Dopa and the other commercially available amino acids were obtained from 'Roche'. The  $d(+)$ -dopa,  $dl$ -*N*-methyldopa and  $dl$ -*N*-methyltyrosine (synthetic surinamine) were prepared by Prof. C. R. Harington. Hydroxytyramine hydrochloride was obtained from Messrs E. Merck; in a few experiments a sample of the hydrobromide from the collection of the late Prof. G. Barger was used, which I obtained from Prof. J. H. Burn. I should like to express my indebtedness for these valuable gifts.

## EXPERIMENTS

The decarboxylation of  $l(-)$ -dopa in extracts from mammalian kidney is a thermolabile enzymic reaction which is abolished by boiling. In agreement with Holtz, Heise & Lüdtke [1938] we have found that 1 mol.  $\text{CO}_2$  per mol. substrate is formed in the reaction. This is illustrated by the following experiment:

Exp. 1. Decarboxylation of  $l(-)$ -dopa by extract from rhesus kidney. Gas phase:  $\text{N}_2$ ; temp.  $37.5^\circ \text{C}$ .

Flask no.	1	2
Main flask	1.6 c.c. rhesus kidney extract	1.6 c.c. rhesus kidney extract
Side bulb	0.4 c.c. water	0.4 c.c. $M/100 \text{ } l(-)$ -dopa solution
Inner tube	0.3 c.c. $N/1 \text{ H}_2\text{SO}_4$	0.3 c.c. $N/1 \text{ H}_2\text{SO}_4$

The experiment was started by tipping into the main flasks the contents of the side bulbs; readings were taken until no further evolution of gas occurred. 18 min. after the start of the reaction the  $\text{H}_2\text{SO}_4$  was spilled into the main flask and the total bound  $\text{CO}_2$  determined.

	Flask no. 1	Flask no. 2	Difference (2-1)
c.mm. $\text{CO}_2$ set free during experiment	2.0	48.5	46.5
c.mm. $\text{CO}_2$ set free after acidifying	16.0	56.5	40.5
Total $\text{CO}_2$ formed		87.0 c.mm.	
Expected formation according to equation (1)		89.5 c.mm.	

Almost one-half of the total  $\text{CO}_2$  formed was retained in the solution. Fig. 1 shows the rate of  $\text{CO}_2$  formation in this experiment. Curve *a* is drawn from the

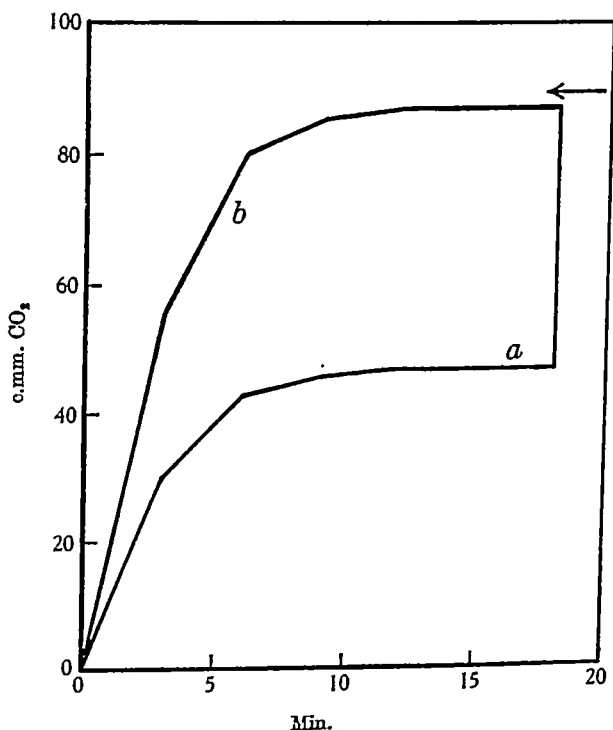


Fig. 1. Time course of formation of  $\text{CO}_2$  in rhesus kidney extract after adding *l*(-)-dopa. Abscissa: time in min.; Ordinate: c.mm.  $\text{CO}_2$ . Curve *a* gives the liberation of  $\text{CO}_2$  during the experiment and the amount liberated after acidifying; curve *b* gives the time course of the reaction, corrected for the retention of  $\text{CO}_2$ . The arrow denotes 1 mol.  $\text{CO}_2$  per mol. *l*(-)-dopa.

actual readings obtained and curve *b* gives the time course of the reaction corrected for the retention of  $\text{CO}_2$ , on the assumption that the retention remained unchanged during the experiment; such an assumption appears justified and curve *b*, therefore, may be taken as a satisfactorily accurate picture of the time course of the reaction.

In the experiment on the human kidney (case C) in which the manometer flasks were set up in the same way as in exp. 1, the amounts of  $\text{CO}_2$  found were as follows:

	Flask no. 1	Flask no. 2	Difference (2-1)
c.mm. $\text{CO}_2$ set free in 17 min.	-1.5	47.5	49
c.mm. $\text{CO}_2$ set free after acidifying	33	72.5	39.5
c.mm. $\text{CO}_2$ formed		83.5	
Expected for complete decarboxylation		89.5 c.mm.	

*Effect of dialysis.* The activity of the extracts was not affected by dialysis. Pig's kidney extract was dialysed in a cellophane tube against distilled water. The dialysis was carried out in a refrigerator at about  $+1^\circ \text{C}$ . and the distilled water was changed several times. After 40 hr. dialysis  $M/2$  phosphate was added to restore the initial phosphate concentration. The decarboxylase activity was compared with that of an undialysed sample of the same extract. During dialysis and by the addition of the  $M/2$  phosphate buffer, the volume of the dialysed sample had increased, and the undialysed sample was diluted to the same extent before measuring the enzymic activity. There was no difference in the rate of decarboxylation by the two samples.

*Distribution and activity.* From the initial rate of decarboxylation of different tissue extracts the activity was calculated as  $\text{dopa-}q_{\text{CO}_2}$ , i.e. the amount of  $\text{CO}_2$  in c.mm. formed per mg. fresh tissue per hr. The figures so obtained give only an approximate value of the decarboxylating activity of the tissues, since there is an unavoidable loss of active material in the preparation of the extracts. In addition; in a few instances the rate of the reaction fell off rather early in the experiment. There is thus the possibility that the actual values for the initial rates are slightly higher than those taken for the determination of the  $q_{\text{CO}_2}$  in Table 1. They should be taken as minimum values. In the last column of the table the amounts of hydroxytyramine formed per g. tissue per hr. are given. The calculation is based on the fact that 1 millimol  $\text{CO}_2$  ( $=22,400$  c.mm.) is equivalent to 153 mg. hydroxytyramine. A value of  $\text{dopa-}q_{\text{CO}_2}$  of 1.0 therefore corresponds to a formation of  $\frac{153 \times 1000}{22400} = 6.83$  mg. hydroxytyramine by 1 g. tissue per hr.

The material from the human kidney, case A, did show that there was a formation of  $\text{CO}_2$  on addition of  $l(-)$ -dopa, but the accuracy of the determination cannot be considered as satisfactory, and the result is not included in Table 1 which gives the results obtained from experiments with extracts B and C.

Table 1 shows the presence of the enzyme in the liver and kidney of all the mammals examined, but its distribution varied widely in different species. We can further confirm the observation of Holtz, Reinhold & Credner [1939] of the absence of the enzyme from frog's kidney (one experiment). Contrary to their findings we obtained a small, but definite formation of  $\text{CO}_2$  with

TABLE 1. Dopa decarboxylase activity of tissue extracts

Species	Organ	Number of expts.	Dopa- $q_{CO_2}$	mg. hydroxytyramine formed per hr. and g. fresh tissue
Guinea-pig	Liver	9	0.470-0.910	3.20-6.20
	Kidney	3	1.860-3.390	12.70-49.60
Pig	Kidney	3	0.310-2.000	2.10-13.70
Cat	Liver	2	0.067, 0.069	0.46, 0.47
	Kidney	2	0.176, 0.186	1.20, 1.27
Dog	Liver	3	0.018-0.044	0.12-0.30
	Kidney	3	0.024-0.043	0.18-0.29
Rhesus	Liver	1	0.130	0.79
	Kidney	2	1.550, 2.300	10.60, 15.70
Man	Kidney	2	0.200, 0.940	1.37, 6.42
<i>Rana temp.</i>	Liver*	3	0.031-0.080	0.21-0.55
	Kidney*	1	0	0

\* These expts. were carried out at 18.5-20.5° C.

extracts from frog's liver. In this connexion it is interesting to note that the enzyme does not occur in the liver, the 'kidneys' and the posterior salivary glands of *Sepia officinalis* [Blaschko, 1941].

**Cyanide inhibition.** Whereas amine oxidase is known to be cyanide-insensitive, dopa decarboxylase is inhibited by cyanide [Holtz, Heise & Lüdtke, 1938]. In an experiment with guinea-pig's liver extract the rate of decarboxylation with and without cyanide was determined; for the first 20 min. the rate of the reaction was almost linear; during this period the inhibition was 72.5% in  $10^{-3}M$  HCN; in  $10^{-4}M$  HCN there was no inhibition.

The inhibition was found to be completely reversible and independent of the time the cyanide had been in contact with the enzyme. Cyanide was added to the extract and the flask was incubated for 100 min. with alkali present in the inner tube. All the cyanide distilled into the alkali, and at the end of the incubation period the cyanide inhibition was abolished. This is shown in the following experiment:

*Exp. 2.*

Flask no.	...	1	2	3
Main flask		1.4 c.c. guinea-pig liver extract + 0.2 c.c. water	1.4 c.c. guinea-pig liver extract + 0.2 c.c. <i>M</i> /50 HCN	1.4 c.c. guinea-pig liver extract + 0.2 c.c. <i>M</i> /50 HCN
Side bulb		0.4 c.c. <i>M</i> /100 l (-)-dopa	0.4 c.c. <i>M</i> /100 l (-)-dopa	0.4 c.c. <i>M</i> /100 l (-)-dopa
Inner tube		0.3 c.c. <i>N</i> /1 H <sub>2</sub> SO <sub>4</sub>	0.3 c.c. <i>N</i> /1 KOH	0.3 c.c. <i>N</i> /1 H <sub>2</sub> SO <sub>4</sub>

After 100 min. incubation at 37.5° C. in  $N_2$ , the alkali was removed from the inner tube of flask 2 and replaced by 0.3 c.c.  $N/1$   $H_2SO_4$ . (Each of the three flasks had a control with 0.4 c.c. water in the side bulb.) The reaction was then started. The amount of  $CO_2$  formed in the first 10 min. was:

Flask no.	...	1	2	3
c.mm. $CO_2$		65	61.5	18
Inhibition %		—	5.5	72

In exp. 3 two manometer flasks (nos. 2 and 3) had the same cyanide concentration; in one flask (no. 2) the cyanide was added to the extract in the main flask and was, therefore, allowed to act on the enzyme during the preparation of the experiment and the period of temperature equilibrium. In the other flask (no. 3) the same amount of cyanide was filled into the side bulb; it was mixed with the enzyme only at the start of the reaction. The figures given show that the initial rate of the reaction was the same in both flasks; the inhibition had not increased in strength during the incubation of the enzyme with cyanide:

Exp. 3.		1	3
Flask no.	...	1	3
Main flask	1.6 c.c. guinea-pig liver extract	1.6 c.c. guinea-pig liver extract + 0.2 c.c. $M/50$ HCN	1.6 c.c. guinea-pig liver extract
Side bulb	0.4 c.c. $M/100$ l(-)-dopa	0.2 c.c. $M/50$ l(-)-dopa	0.2 c.c. $M/50$ l(-)-dopa + 0.2 c.c. $M/50$ HCN
Inner tube	0.3 c.c. $N/1$ $H_2SO_4$	0.3 c.c. $N/1$ $H_2SO_4$	0.3 c.c. $N/1$ $H_2SO_4$

Each flask had one control flask in which the dopa solution was replaced by water. The amounts of  $CO_2$  (uncorr.) in c.mm. formed in the first 15 min. were:

Flask no.	...	1	2	3
		30	7	7

These results show that the inhibition by cyanide of dopa decarboxylase resembles that of cell respiration and of metal-containing enzymes, but differs from that of xanthine oxidase, the inhibition of which is irreversible and progressive [Dixon & Keilin, 1936].

*Other inhibitors.* Carbon monoxide had no inhibitory action; it was used instead of nitrogen at a pressure of one atmosphere. Sodium sulphide caused slight inhibition: in  $M/50$   $Na_2S$  it amounted to 21%. Sodium azide did not inhibit the enzyme at a concentration of  $M/5000$  and  $M/500$ ; its effect was examined at pH 7.4 and 6.5.

Suramine (antrypol B.D.H.) and trypan blue inhibited the enzyme. In  $M/1000$  suramine the enzyme was completely inhibited. In  $M/10,000$  suramine the initial rate during the first 5 min. was about 57% of that without suramine; the inhibition was progressive and the reaction came to a standstill within 30 min. Trypan blue also inhibited: the inhibition was 8% at  $M/10,000$ , and 33% at  $M/1000$ . These two substances have a similar inhibitory action on the mammalian decarboxylase for l(-)-histidine [Werle, 1940].

*Inhibition by hydroxytyramine.* Hydroxytyramine, the end-product of the reaction, also acted as a slight inhibitor. This fact may account, at least partly, for the slowing down of the reaction in the course of an experiment, during which hydroxytyramine is formed and accumulates. The effect of hydroxytyramine is shown in exp. 4.

TABLE 1. Dopa decarboxylase activity of tissue extracts

Species	Organ	Number of expts.	Dopa- $\gamma$ CO <sub>2</sub>	mg. hydroxytyramine formed per hr. and g. fresh tissue
Guinea-pig	Liver	9	0.470-0.910	3.20-6.20
	Kidney	3	1.860-3.390	12.70-49.60
Pig	Kidney	3	0.310-2.000	2.10-13.70
Cat	Liver	2	0.087, 0.089	0.46, 0.47
	Kidney	2	0.176, 0.186	1.20, 1.27
Dog	Liver	3	0.018-0.044	0.12-0.30
	Kidney	3	0.024-0.043	0.16-0.29
Rhesus	Liver	1	0.130	0.79
	Kidney	2	1.550, 2.300	10.60, 15.70
Man	Kidney	2	0.200, 0.940	1.37, 6.42
<i>Rana temp.</i>	Liver*	3	0.031-0.080	0.21-0.55
	Kidney*	1	0	0

\* These expts. were carried out at 18.5-20.5° C.

extracts from frog's liver. In this connexion it is interesting to note that the enzyme does not occur in the liver, the 'kidneys' and the posterior salivary glands of *Sepia officinalis* [Blaschko, 1941].

**Cyanide inhibition.** Whereas amine oxidase is known to be cyanide-insensitive, dopa decarboxylase is inhibited by cyanide [Holtz, Heise & Lüdtkke, 1938]. In an experiment with guinea-pig's liver extract the rate of decarboxylation with and without cyanide was determined; for the first 20 min. the rate of the reaction was almost linear; during this period the inhibition was 72.5% in  $10^{-3}M$  HCN; in  $10^{-4}M$  HCN there was no inhibition.

The inhibition was found to be completely reversible and independent of the time the cyanide had been in contact with the enzyme. Cyanide was added to the extract and the flask was incubated for 100 min. with alkali present in the inner tube. All the cyanide distilled into the alkali, and at the end of the incubation period the cyanide inhibition was abolished. This is shown in the following experiment:

*Exp. 2.*

Flask no.	...	1	2	3
Main flask		1.4 c.c. guinea-pig liver extract + 0.2 c.c. water	1.4 c.c. guinea-pig liver extract + 0.2 c.c. <i>M</i> /50 HCN	1.4 c.c. guinea-pig liver extract + 0.2 c.c. <i>M</i> /50 HCN
Side bulb		0.4 c.c. <i>M</i> /100 l (-)-dopa	0.4 c.c. <i>M</i> /100 l (-)-dopa	0.4 c.c. <i>M</i> /100 l (-)-dopa
Inner tube		0.3 c.c. <i>N</i> /1 H <sub>2</sub> SO <sub>4</sub>	0.3 c.c. <i>N</i> /1 KOH	0.3 c.c. <i>N</i> /1 H <sub>2</sub> SO <sub>4</sub>

After 100 min. incubation at 37.5° C. in N<sub>2</sub>, the alkali was removed from the inner tube of flask 2 and replaced by 0.3 c.c.  $N/1$  H<sub>2</sub>SO<sub>4</sub>. (Each of the three flasks had a control with 0.4 c.c. water in the side bulb.) The reaction was then started. The amount of CO<sub>2</sub> formed in the first 10 min. was:

Flask no.	...	1	2	3
c.mm. CO <sub>2</sub>	...	65	61.5	...
Inhibition %	...	—	5.5	...

In exp. 3 two manometer flasks (nos. 2 and 3) had the same cyanide concentration; in one flask (no. 2) the cyanide was added to the extract in the main flask and was, therefore, allowed to act on the enzyme during the preparation of the experiment and the period of temperature equilibrium. In the other flask (no. 3) the same amount of cyanide was filled into the side bulb; it was mixed with the enzyme only at the start of the reaction. The figures given show that the initial rate of the reaction was the same in both flasks; the inhibition had not increased in strength during the incubation of the enzyme with cyanide:

Exp. 3.		1	2	3
Flask no.	...	1	1	3
Main flask	1.6 c.c. guinea-pig liver extract	1.6 c.c. guinea-pig liver extract + 0.2 c.c. $M/50$ HCN	1.6 c.c. guinea-pig liver extract	
Side bulb	0.4 c.c. $M/100$ $l(-)$ -dopa	0.2 c.c. $M/50$ $l(-)$ -dopa	0.2 c.c. $M/50$ $l(-)$ -dopa + 0.2 c.c. $M/50$ HCN	
Inner tube	0.3 c.c. $N/1$ $H_2SO_4$	0.3 c.c. $N/1$ $H_2SO_4$	0.3 c.c. $N/1$ $H_2SO_4$	

Each flask had one control flask in which the dopa solution was replaced by water. The amounts of  $CO_2$  (uncorr.) in c.mm. formed in the first 15 min. were:

Flask no.	...	1	2	3
		30	7	7

These results show that the inhibition by cyanide of dopa decarboxylase resembles that of cell respiration and of metal-containing enzymes, but differs from that of xanthine oxidase, the inhibition of which is irreversible and progressive [Dixon & Keilin, 1936].

*Other inhibitors.* Carbon monoxide had no inhibitory action; it was used instead of nitrogen at a pressure of one atmosphere. Sodium sulphide caused slight inhibition: in  $M/50$   $Na_2S$  it amounted to 21%. Sodium azide did not inhibit the enzyme at a concentration of  $M/5000$  and  $M/500$ ; its effect was examined at pH 7.4 and 6.5.

Suramine (antypol B.D.H.) and trypan blue inhibited the enzyme. In  $M/1000$  suramine the enzyme was completely inhibited. In  $M/10,000$  suramine the initial rate during the first 5 min. was about 57% of that without suramine; the inhibition was progressive and the reaction came to a standstill within 30 min. Trypan blue also inhibited: the inhibition was 8% at  $M/10,000$ , and 33% at  $M/1000$ . These two substances have a similar inhibitory action on the mammalian decarboxylase for  $l(-)$ -histidine [Werle, 1940].

*Inhibition by hydroxytyramine.* Hydroxytyramine, the end-product of the reaction, also acted as a slight inhibitor. This fact may account, at least partly, for the slowing down of the reaction in the course of an experiment, during which hydroxytyramine is formed and accumulates. The effect of hydroxytyramine is shown in exp. 4.

Exp. 4. Total volume: 2.0 c.c. 1.0 c.c. extract of guinea-pig (liver + kidney). Initial concentration of *l*(-)-dopa: *M*/500.

	Without hydroxytyramine	With <i>M</i> /50 hydroxytyramine
o.mm. CO <sub>2</sub> formed in 10 min.	38.5	10.5
Inhibition %	—	71

**Stereospecificity.** Holtz, Heise & Lüdtke [1938] have found that the amounts of hydroxytyramine formed from *l*(-)-dopa were twice as large as those from *dl*-dopa, and they concluded that the dextrorotatory stereoisomeride was not attacked by the decarboxylase. This is, in fact, the case: using *d*(+)-dopa as substrate, we found no formation of CO<sub>2</sub> with extracts from guinea-pig's liver and pig's kidney.

**Substrate specificity.** A number of other amino acids (shown in Table 2) were tested with some of the extracts. In no case was there any formation of CO<sub>2</sub>.

TABLE 2. Observations on various amino acids  
+ signifies formation of CO<sub>2</sub>; - signifies no formation of CO<sub>2</sub>

Substrate	Pig Kidney	Guinea-pig		Rhesus	
		Liver	Kidney	Liver	Kidney
<i>l</i> (-)-dopa	+	+	+	+	+
<i>d</i> (+)-dopa	-	-	.	.	.
<i>dl</i> -N-methyldopa	-	-	.	.	-
<i>l</i> (-)-tyrosine	-	.	.	-	-
<i>dl</i> -N-methyltyrosine (synthet. surinamine)	.	-	.	.	.
<i>dl</i> -phenylalanine	.	.	-	.	.
<i>l</i> (-)-tryptophan	.	-	.	.	.
<i>l</i> (-)-histidine	-	-	-	-	-
<i>d</i> (+)-histidine	-	.	.	.	.
<i>dl</i> -alanine	.	.	-	.	.
<i>dl</i> -proline	.	.	-	.	.
<i>dl</i> -hydroxyproline	.	.	-	.	.
<i>dl</i> -serine	.	.	-	.	.
<i>l</i> (-)-leucine	.	.	-	.	.

**Presence of amine oxidase in human kidney.** Part of the extract prepared from kidney *C* was used for determination of its amine oxidase activity. The manometer flasks were set up as follows:

Main flask	1.6 c.c. extract + 0.2 c.c. <i>M</i> /4 semicarbazide hydrochloride (neutralized)
Side bulb	0.2 c.c. <i>M</i> /4 amine hydrochloride
Inner tube	0.3 c.c. <i>N</i> /1 KOH
Gas	O <sub>2</sub> ; t. 37.5° C.

The amines used were tyramine, *iso*-amylamine and (-)-*p*-sympatol. The amounts of O<sub>2</sub> (in c.mm.) consumed in 9 min. were:

Without substrate	Additional uptake with		
	Tyramine	<i>iso</i> Amylamine	(-)- <i>p</i> -Sympatol
32.5	51.5	70.5	26

Taking the oxygen consumption with tyramine as 100, the relative rates of oxidation were:

With <i>iso</i> amylamine	137
With (-)- <i>p</i> -sympatol	50



## DISCUSSION

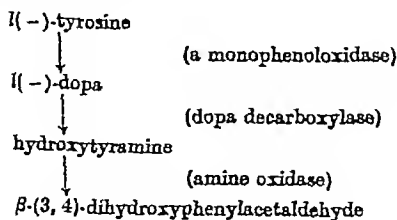
Amine oxidase, the enzyme which oxidizes hydroxytyramine has a great number of substrates. Holtz, Heise & Lüdtke [1938] therefore discussed whether the oxidase for the amino acids of the naturally occurring *l*-series was composed of two enzymes, namely an amino acid decarboxylase and amine oxidase, in other words, whether in the breakdown of amino acids the amine appeared normally as an intermediary metabolite.

Table 2 shows that of the amino acids tried only dopa is decarboxylated. This renders it unlikely that decarboxylation and subsequent oxidation is the main pathway of amino acid breakdown. In fact, for some amino acids of the *l*-series, e.g. *l*(-)-aspartic and *l*(+)-glutamic acids, the formation of the corresponding ketonic acid has been established [Krebs, 1933].

Our results do not rule out the possibility of decarboxylation of other amino acids in the mammalian body. For instance, a decarboxylase for *l*(-)-cysteic acid occurs in the liver of the dog [Blaschko, 1942]. Decarboxylases for *l*(-)-histidine and *l*(-)-tyrosine have also been described [Werle & Menniken, 1937; Holtz, 1937; Holtz, Heise & Spreyer, 1938], but the rates of decarboxylation are too small to be measured by manometric methods.

The specificity of the decarboxylase suggests that the natural substrate of the enzyme is dopa, and that this amino acid has a wider distribution in nature than is at present realized. It may be an intermediary metabolite; its formation from tyrosine is, in fact, suggested by the following observations on tyrosinosis and on melanin formation. In tyrosinosis *l*(-)-dopa was found to be excreted in the urine after oral administration of *l*(-)-tyrosine [Medes, 1932]. If this condition were due to an inability of the organism to complete the normal breakdown of tyrosine, it would indicate *l*(-)-dopa as a normal intermediary. Dopa is the first product in the formation of melanin from tyrosine by the tyrosinase of invertebrates and plants [Raper, 1926; Evans & Raper, 1937], and it is believed to play a similar role in mammalian melanoblasts [Bloch, 1916].

These findings suggest for the breakdown of tyrosine a pathway different from those hitherto described, viz.:



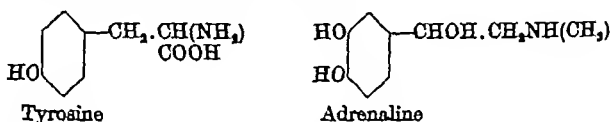
The existence of such a pathway might explain the failure of Felix, Zorn & Dirr-Kaltenbach [1937] to find *p*-hydroxyphenylpyruvic acid when *l*(-)-

tyrosine was incubated with tissue extracts, but it would not explain why no ammonia was found [see also Bernheim & Bernheim, 1934; Bernheim, 1935].

A comparison of our own data for *l*(-)-dopa with those for the decarboxylation of *l*(-)-tyrosine by *Streptococcus faecalis* [Gale, 1940] shows an activity of the mammalian tissue of less than 1% of that of the bacteria under optimal conditions. The *pH* optimum of the mammalian enzyme is between 7.5 and 6.5, that of the bacterial decarboxylases between 5.0 and 4.5. Both the bacterial and the mammalian enzymes show a high degree of substrate specificity and are stereospecific for the naturally occurring stereoisomeride.

Our results have some bearing on the problem of the formation of adrenaline, sympathin E and pressor substances in the ischemic kidney.

*Formation of adrenaline.* It is usually believed that adrenaline is formed from tyrosine, although the intermediary stages are unknown. It has been pointed out that dopa decarboxylase may catalyse one reaction in the synthesis of adrenaline from tyrosine [Blaschko, 1939].



At least four reactions must occur in this process:

- (a) Introduction of the second phenolic hydroxyl group;
- (b) Decarboxylation;
- (c) Introduction of the side chain hydroxyl group;
- (d) *N*-methylation.

If the reaction catalysed by *l*(-)-dopa decarboxylase represents an intermediary step in adrenaline synthesis, a number of conclusions as to the sequence of these four reactions are possible:

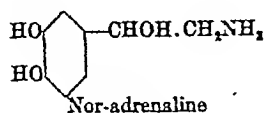
(1) Reaction (a) is probably the first reaction to take place; evidence that formation of dopa from tyrosine occurs has been given above.

(2) Reaction (b), the reaction studied in this paper, must precede (d) and follow (a), since both tyrosine and *N*-methyl-dopa are not decarboxylated at a measurable rate. *N*-Methyl-dopa has been shown not to give rise to adrenaline in the perfused suprarenal glands of the cow [Heard & Raper, 1933].

In the sequence of reactions outlined above, tyramine does not appear as an intermediary. Formation of adrenaline from tyramine has been said to occur [Schuler & Wiedemann, 1935]; these results have not generally been accepted [Holtz, 1937]. But Devine [1940] has found an increased adrenaline content in suprarenal glands incubated with phenylethylamine.

The stage at which reaction (c) occurs cannot be fixed with the evidence at present available; it is unknown whether (3,4)-dihydroxyphenylserine is decarboxylated [Rosenmund & Dornsaft, 1919].

*Decarboxylase and sympathin E.* The fact that the body contains an enzyme that forms a primary sympathomimetic amine, but not the corresponding secondary amine, may explain some observations on sympathin. The primary amines are imperfect sympathomimetics; their action is mainly excitatory and they do not possess all the inhibitory effects of adrenaline [Barger & Dale, 1910]. The sequence of reactions outlined above would require a primary amine to be the precursor of adrenaline, the one most closely related being nor-adrenaline.



The possibility of the identity of sympathin E with nor-adrenaline was suggested by Bacq [1934], and supported by observations of Stehle & Ellsworth [1937] and of Melville [1937]. Greer, Pinkston, Baxter & Brannon [1938] compared the effects of stimulation of the hepatic sympathetic nerves with those of intravenous injections of either adrenaline or nor-adrenaline. The latter was found to reproduce the action of 'liver sympathin' more faithfully than adrenaline. Cannon & Rosenblueth [1935] found it difficult to accept the idea of a demethylation of adrenaline. Since a primary sympathomimetic amine can arise directly in the body, this demethylation need no longer be assumed to occur. The experiments described in this paper suggest that the primary amine is formed first and that the secondary amine arises from it by N-methylation. Such reactions are known to occur in the body, e.g. in the synthesis of creatine [Borsook & Dubnoff, 1940; Bloch & Schoenheimer, 1941; Du Vigneaud, Cohn, Chandler, Schenk & Simmonds, 1941].

*Formation of pressor substances in the ischaemic kidney.* The decarboxylase forms a pressor substance from the pharmacologically inert dopa. This is of interest in connexion with the formation of pressor substances in the ischaemic kidney [Goldblatt, 1937; Verney & Vogt, 1938]. If hydroxytyramine were formed in the kidney as an intermediary metabolite it would normally be destroyed by amine oxidase, but under conditions of oxygen lack one would expect it to accumulate. Partial pressures of oxygen of the order that are found in the living tissue are, in fact, a limiting factor for the action of amine oxidase [Kohn, 1937]. In the ischaemic kidney some of the amine formed might therefore escape into the circulation. The ischaemic mammalian kidney perfused with *l*(-)-dopa releases a pressor substance having the properties of hydroxytyramine [Bing, 1941], whereas in the frog there occurs no rise in blood pressure when the kidneys are made ischaemic [Vogt, 1940]. This could be explained by the absence of the decarboxylase in the frog's kidney.

## SUMMARY

1. In liver and kidney extracts the activity of *l*(-)-dopa decarboxylase is determined manometrically by measuring the formation of carbon dioxide under anaerobic conditions. In mammals, the enzyme was found to be present in the extracts of all the species examined, including the human kidney. It was present in the liver, but not in the kidney, of frogs.

2. The enzyme is inhibited reversibly by cyanide. The inhibition is not progressive. The enzyme is not inhibited by carbon monoxide and sodium azide, but by suramine and hydroxytyramine.

3. Extracts containing the enzyme do not decarboxylate at a comparable rate: *d*(+)-dopa, *dl*-N-methyldopa, *l*(-)-tyrosine, *dl*-N-methyltyrosine, *l*(-)-phenylalanine as well as a number of other amino acids.

4. These observations suggest a new pathway of tyrosine breakdown *via l*(-)-dopa and hydroxytyramine, but they give no support to the suggestion that *l*-amino acids are generally metabolized by decarboxylation and subsequent oxidative deamination.

5. The significance of these findings in connexion with the formation of adrenaline, sympathin E and pressor substances in the ischaemic kidney is discussed.

6. Extracts from human kidney contain the enzyme amine oxidase.

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## THE SIGNIFICANCE OF URINARY CALCIUM, MAGNESIUM AND PHOSPHORUS

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*(Received 6 June 1942)*

Some elements, such as sodium, are excreted almost entirely in the urine; others, such as iron, in the faeces. Calcium and magnesium, however, are excreted in important amounts by both routes, and it has always been a problem to decide what is the proper function and significance of each. In 1939 McCance & Widdowson demonstrated that when Ca and Mg salts were injected intravenously into normal human adults, their rate of excretion by the kidney increased, and the excess eliminated by this organ corresponded closely to the amount which had been injected. There was no increased excretion by the bowel. They argued that, although Ca and Mg might be excreted into the gut in the intestinal secretions, and fail to be reabsorbed, this loss represented a fixed charge upon the body, and was to be contrasted with the renal excretion, which their results had shown to vary in accordance with physiological requirements. It was admitted that in certain diseases such as thyrotoxicosis there might be an increase in the amount of Ca eliminated through the bowel, but it was maintained that this did not in any way contradict their views as to the real partition of function between the kidney and the gut. The renal excretion also may be altered in thyroid disease, but it now seems that this change is due to the direct action of the thyroid hormone on the renal threshold for Ca [Robertson, 1942]. It is, therefore, due to pathological lesions in the kidney itself, and consequently its discussion really lies outside the scope of the present paper, which is concerned with the function of the normal organ.

The data now to be reported have been obtained in metabolism experiments which have been carried out on normal persons in the last two years. Full descriptions of the dietary and other technical arrangements have already been given [McCance & Widdowson, 1942*a*, *b*]. It is only necessary to state here that the experiments were designed to study the effects of wheaten breads of various kinds upon the absorption of Ca and other minerals, and in each experiment the flour undergoing investigation furnished 40-50% of the total

calories. Each experiment lasted for at least 14 and usually 21 days, together with the usual 3 or 4 preliminary and 1 or 2 after days. Consequently, the daily fluctuations in urinary excretion, and the short-term variations in faecal output which have been so much discussed [Ascham, 1930-1; Nicholls & Nimalasuriya, 1939], were very largely eliminated.

TABLE 1. The response of the kidney to a varying absorption of calcium

Subject	Nature of the diet modifying absorption: brown bread		Nature of the diet modifying absorption: dephytinized brown bread		Nature of the diet modifying absorption: white bread		Nature of the diet modifying absorption: white bread with added Ca salts	
	Absorp- tion mg./day	Urine mg./day	Absorp- tion mg./day	Urine mg./day	Absorp- tion mg./day	Urine mg./day	Absorp- tion mg./day	Urine mg./day
E.B.	89	153	231	222	250	240	403	354
N.C.	74	153	279	167	232	258	—	—
N.J.	37	46	67	82	111	107	—	—
H.E.	57	152	169	217	192	234	—	—
A.M.	7	60	120	165	142	123	273	153
W.Y.	23	133	123	147	183	187	—	—
Average	35	116	165	170	185	191	338	254

Table 1 gives some information about the absorptions and urinary excretions of Ca. By the term 'absorption' is meant the amount of Ca in the food minus the amount in the faeces. If this figure is equal to the amount of Ca in the urine, the person is neither gaining nor losing Ca, and is said to be 'in balance'. The absorptions from the brown-bread diets were low because of the phytic acid in these diets. Hydrolysis of the phytates in the flour allowed the absorptions to rise, as did a change to a white-bread diet, and the addition of Ca salts to the white-bread diets produced the highest absorptions of all. On the dephytinized and white-bread diets, i.e. when the absorptions were moderate, the urinary Ca averaged a figure little different from that for the absorptions. On the brown-bread diets the urinary outputs fell, but not by enough to compensate for the reduced absorptions, and consequently all the subjects were in negative balance. The urinary outputs during these experiments were being maintained at the expense of the bodily reserves. When E.B.'s and A.M.'s absorptions were raised by adding Ca to the diets, their urinary excretions also rose, but by less than the absorptions, and both the subjects went into positive balance. The Ca absorbed but not excreted was probably deposited in the skeleton. It is quite evident that the amount of Ca in the urine depended partly upon the amount absorbed, and that it varied directly with it. It is also clear, however, that the urinary excretions of Ca did not follow to their extreme limits the fluctuations in the intestinal absorptions. Nevertheless, in normal persons it has been found that changes in the urinary excretions of Ca may be used as an index of changes in the amount of Ca absorbed. The determination of Ca in the urine, therefore, should never be omitted when absorp-

tions are under investigation, for it offers valuable confirmation of the movements of the latter. Technically, moreover, it is a comforting corroboration, for the absorptions represent differences—often small—between two larger numbers, one of which can never be measured with real accuracy, while the Ca in the urine is obtained by direct determination without any mensural uncertainties.

It would have been satisfactory to have been able to demonstrate changes in the serum Ca which might have initiated, even if they were not wholly responsible for, the changes in renal excretion, but this has not been done. The changes may be very slight, and comparable with the insignificant changes in serum sodium, chloride and total solids which precede huge changes in the rate of excretion of salt and water. True, these excretions are known to be regulated by ductless glands, but there is some evidence now that the renal excretion of P is under the direct control of the parathyroid hormone and also of vitamin D [Harrison & Harrison, 1941]. Something similar *may* be true of Ca. It has yet to be proved, however, that any vitamin or hormone [Robertson, 1942] has anything to do with the physiological changes in urinary Ca which are now being discussed.

TABLE 2. The response of the kidney to a varying absorption of magnesium

Subject	Nature of the diet modifying absorption: brown bread (1) or white bread with added sodium phytate (2)		Nature of the diet modifying absorption: white bread		Nature of the diet modifying absorption: dephytinized brown bread		Nature of the diet modifying absorption: white bread with added MgCO <sub>3</sub>	
	Absorption mg./day	Urine mg./day	Absorp- tion mg./day	Urine mg./day	Absorp- tion mg./day	Urine mg./day	Absorp- tion mg./day	Urine mg./day
N.C.	47 (1)	120	163	152	209	175	—	—
N.J.	166 (1)	171	—	—	209	202	—	—
E.B.	86 (2)	90	128	132	215	170	240	176
N.K.	129 (2)	125	218	229	—	—	—	—
P.S.	92 (2)	100	169	156	—	—	237	210
R.W.	83 (2)	72	103	103	—	—	143	135

Table 2 gives data about Mg. The small absorptions from brown bread and from white bread with added sodium phytate were due to the phytic acid precipitating the Mg in the intestine. Brown bread, however, contains much more Mg than white, and enzymatic hydrolysis of the phytic acid in brown flour allowed freer absorption to take place, and the same result was achieved by adding Mg salts to white bread. Except for one subject, N.C., the urinary excretions fell with the absorptions to their lowest levels. At the highest absorptions N.C., E.B. and P.S. went into positive balance. So far as the accuracy of the methods allow one to state, N.J. and R.W.'s urinary excretions followed their intestinal absorptions even to the high levels of the latter.

The urinary excretions of Mg, therefore, may be taken to reflect the absorptions of the metal, and the practical considerations which applied to Ca apply



equally to Mg. It is possible, although not demonstrable with certainty from the data in Tables 1 and 2, that the urinary excretions of Mg will be found to equal the absorptions over wider excursions of the latter than do the urinary excretions of Ca.

Similar data can be obtained from a study of P, and similar reasoning may be applied.

### DISCUSSION

If we suppose that in these, as in so many other, aspects of renal activity the function of the kidney is solely to regulate the constancy of the internal environment, and if further we regard the skeleton as having very much the same function where Mg [Duckworth & Godden, 1941], Ca and P are concerned, then we can explain all the observations on normal persons. Minor fluctuations in absorption, and hence in the serum chemistry, tend to be corrected by the kidney alone, but if the increment is more considerable, Ca is deposited in the skeleton and positive balances ensue. When the absorption falls to a very low level, or is replaced by a loss into the intestine (see Table 1), nothing that the kidney alone can do will maintain the stability of the internal environment, and this is achieved only by a liberation of salts from the skeleton. An interesting point arises here. Owing to this role of the skeleton, the constancy of the internal environment and the excretion of Ca by the kidney can each be regarded as tending to confer stability upon the other. It is this buffering action of the skeleton which explains why gross fluctuations in absorption are not followed by equally gross excursions in urinary excretion.

The present theory in no way conflicts with the well-established fact that the urinary Ca can be increased out of all proportion by an acidosis, or by diseases such as *osteitis fibrosa cystica*, which mobilize Ca from the skeleton. The kidney should be regarded as functioning quite normally in such cases, and attempting as always to stabilize the serum chemistry. It is the skeleton which is at fault and losing Ca so rapidly that the normal fluctuations in intestinal absorption and in the composition of the serum are masked by a rise of Ca in the internal environment. Even working under high pressure, the kidney is unable to excrete the Ca as fast as it is poured into the serum by the skeleton, and the rise in serum Ca, which generally accompanies parathyroid overactivity, may be looked upon as a measure of its inability to do so when the skeleton, usually its ally and partner, has turned against it. When the parathyroid glands are so inactive that Ca is deposited in the skeleton rapidly enough to menace the stability of the internal environment, the kidney may and often does cease to excrete any Ca at all. It can, however, do nothing further, and if Ca continues to be deposited in the skeleton, the serum Ca will continue to fall.

In thyrotoxicosis and myxoedema the Ca metabolism of the skeleton should probably be regarded as normal and that of the kidney as pathological. The low calcium values in the serum are the result of a lowered renal threshold,

and the decalcification of the skeleton is an expression of its efforts to maintain the serum Ca within normal limits.

These views about the role of the kidney in the excretion of Ca are in keeping also with what is known of the metabolism in rickets. The kidney, it is suggested, functions normally in this disease, and excretes Ca according to its level in the internal environment. It is well known that the action of vitamin D is (a) to increase absorption, (b) to increase the deposition of Ca in the bones, and (c) to increase the excretion of Ca by the kidney. It is suggested that (c) is the direct consequence of (a). Table 3 illustrates these activities of vitamin D.

TABLE 3. The effect of vitamin D upon the absorption, deposition and urinary excretion of calcium in a boy with resistant rickets

Vitamin D dosage i.u./day	Ca intake mg./day	Ca absorption mg./day	Ca retention (or deposition) mg./day	Ca in urine mg./day
0	890	415	301	114
90,000	906	761	548	213

The data are taken from an investigation of a child of 8 who was suffering from resistant rickets and required 90,000 i.u. of vitamin D to induce healing. The figures show all three effects and it is suggested that the renal changes are merely the normal response of the kidneys to a rise in the serum Ca.

### SUMMARY

1. The urinary excretions of Ca, Mg and P by normal persons rise and fall with the intestinal absorptions. They do not, however, rise so high or fall so low as the absorptions may do.

2. From the technical point of view, a change in a normal person's urinary excretion should be regarded as an indirect but very valuable confirmation of a change in intestinal absorption.

3. In these, as in so many other, branches of metabolism the function of the kidney is to regulate the stability of the internal environment.

The authors are very grateful to the subjects for their co-operation and to Miss B. Alington for her assistance. The child whose data are given in Table 3 is a patient of Dr J. C. Spence. The Medical Research Council provided a large part of the expenses.

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## THE EFFECT OF ANAESTHESIA ON THE ADRENALINE CONTENT OF THE SUPRARENAL GLANDS

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Numerous observations have been made on the effect of anaesthetics on intestinal and splenic activity, and it has been customary to attribute these effects to the nervous control of these organs rather than to changes in the concentration of circulating adrenaline. Little attention has been paid to the possibility of a secretion of adrenaline from the suprarenal glands resulting from the central action of the anaesthetic, in spite of the observations made by Elliott in 1912. Elliott showed that the adrenaline content of the suprarenal glands of cats is approximately the same on each side. He found that, during anaesthesia, a considerable amount of adrenaline was lost from the normal gland when compared with the gland which was denervated by cutting the splanchnic nerves. Ether, for instance, produced a loss of 50% of the stored adrenaline during a period of 6 hr.

Elliott, however, carried out relatively few experiments with anaesthetics and did not measure the quantities administered. Failing confirmation by subsequent work, his results have been attributed to the excitement during the induction of anaesthesia. Several authors have repeated his observations with varying results [Keeton & Ross, 1919; Kodama, 1923; Fujii, 1924; Marconi & Marco, 1937; Emerson, 1938; Barman, 1939].

In order to determine whether adrenaline secretion does in fact occur during anaesthesia, we have modified Elliott's method so as to exclude the effect of induction and the accompanying excitement. Observations have been made on the effects of three anaesthetics in common use—ether, cyclopropane and pentobarbitone (nembutal).

### METHODS

Cats were used because the adrenaline content of the suprarenals of the two sides is approximately equal in the normal animal. In all animals the induction of anaesthesia was carried out with ether; anaesthesia was maintained either

by the administration of ether, or by that of cyclopropane or pentobarbitone. The splanchnic nerves on one side were cut, but not until the animal was fully anaesthetized with the agent under observation, thus excluding the effect of induction. The abdomen was opened by a mid-line incision, the nerves being cut with the least possible disturbance of the viscera, and the wound was sewn up again. It was from this point that the period of anaesthesia was measured.

The mode of administration differed with the three anaesthetics. After a dose of 1.5 mg. morphine hydrochloride, pentobarbitone was injected intravenously as a 2% solution. Sufficient was given to produce complete abdominal relaxation without respiratory or circulatory depression, and this condition was maintained throughout the experimental period by further small doses of pentobarbitone.

In both the cyclopropane and the ether experiments the animals were artificially respired to ensure a control of the anaesthetic concentrations. For the first 10–15 min. of the cyclopropane anaesthesia, 25% cyclopropane in oxygen was delivered to the cat to obtain a partial gas equilibrium before a closed circuit was set up. The closed system was necessary because mixtures of cyclopropane and oxygen are very explosive. A glass T-piece was tied into the trachea and connected to the respiration pump. This drew anaesthetic mixture from a rigid reservoir and delivered it to the cat. The expired mixture passed through the pump, and from there through a soda-lime canister back to the reservoir. A steady flow of oxygen was added to the reservoir to maintain the pressure at atmospheric, and measured quantities of cyclopropane were added so as to maintain the concentration between 20 and 25%. The concentration was determined by taking samples from the system at half-hourly intervals and absorbing the cyclopropane in concentrated sulphuric acid in a simplified Haldane apparatus.

The ether was administered by a respiration pump connected to a valved trachea tube. This ensured that the animal only breathed anaesthetic mixture without the use of a closed system. The mixture delivered to the pump was regulated by the use of an Oxford Vaporiser No. 1 described by Epstein, Macintosh & Mendelsohn [1941]. In this apparatus the ether vapour is diluted with air and the concentration is measured empirically; it varied when the pump stroke was altered.

At the end of the anaesthetic period both suprarenal glands were removed from the cat and extracted by grinding with acidified saline and sand. After heating to coagulate the protein, the extracts were filtered and made up to a standard volume, either 15 or 16 c.c. The adrenaline content of the two extracts from each cat were compared with each other and with a standard adrenaline solution freshly made from powder in the same acidified saline. The assays were done on spinal cats, bracketing the pressor response to doses of one solution between smaller and larger doses of the other. From the adrenaline content

of the extracts the relative depletion of the innervated gland's stored adrenaline was calculated by expressing the estimated content as a percentage of that of the denervated gland.

## RESULTS

*Ether.* At first we determined the effect of varying periods of ether anaesthesia. In Table 1 the denervated suprarenal is taken as the control with which the innervated gland is compared. In cat no. 1 the splanchnic nerves of one

TABLE 1. The effect of periods of ether anaesthesia on the adrenaline content of the innervated suprarenal gland

Cat no.	Side denervated	Period of anaesthesia	Denervated gland		Innervated gland		% control	Rate of loss %/hr.
			Wt. mg.	Content mg./g.	Wt. mg.	Content mg./g.		
1	L	Induction	204	0.75	149	0.75	100	—
2	—	—	168	1.06	152	1.05	99	—
3	L	2 hr. 20 min.	157	2.67	159	2.34	88	5.14
4	R	1 " 55 "	221	0.54	211	0.47	87	6.78
5	R	2 " 40 "	179	1.29	179	0.83	64	13.5
6	L	4 " 50 "	338	0.98	342	0.70	72	5.79
7	L	5 " 0 "	314	0.48	313	0.38	79	4.20
8	R	4 " 50 "	159	1.08	151	0.83	77	4.76

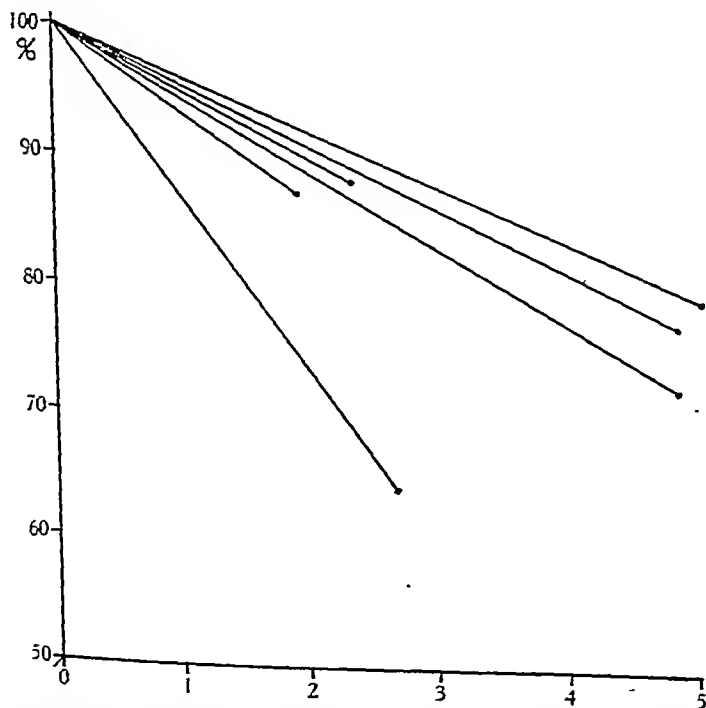


Fig. 1. The loss of adrenaline from the innervated suprarenal glands of six cats during ether anaesthesia. Ordinate: adrenaline content as percentage of that of the denervated glands. Abscissa: duration of anaesthesia in hours.

side were cut by a sterile operation 4 days beforehand. Both the suprarenals were removed immediately after the period of induction which lasted about 15 min.; no difference in adrenaline content was detected. In cat no. 2, both splanchnic nerves were intact, and again there was no significant difference between the two sides. This confirms Elliott's observation that the adrenaline content of the two suprarenal glands in cats is the same, and also shows that the period of induction need not cause more than a negligible secretion. The results obtained in cats nos. 3-8 indicate a progressive loss of adrenaline from the innervated suprarenal gland during anaesthesia. Fig. 1 shows that this loss occurred at approximately the same rate in all except one animal; the average rate of loss for the whole series was 6.7%/hr. A gradual fall of blood pressure was always noticed during the ether anaesthesia, which was very deep, the ether concentration varying between 4 and 8%. Cat no. 5 had a blood pressure of less than 40 mm. Hg for the last hour of the experimental period, and we believe this to be the reason for the excessive adrenaline loss.

As the rate of adrenaline secretion during ether anaesthesia seemed to be constant throughout the experimental period, we fixed the duration of cyclopropane and pentobarbitone anaesthesia at 5 hr. in order to obtain as great a loss of adrenaline from the innervated gland as possible.

*Pentobarbitone.* The results obtained with pentobarbitone are shown in Table 2. The total dose necessary to produce surgical anaesthesia for 5 hr. varied between 19.2 and 51.2 mg./kg. We could not discover any relation between this variation and the amount of adrenaline lost from the suprarenal gland. The blood pressure in these experiments remained normal throughout (100-150 mm. Hg).

TABLE 2. The effect of anaesthesia with pentobarbitone and morphine on the adrenaline content of the innervated suprarenal

Cat no.	Period of anaesthesia	Total dose of pentobarbitone mg./kg.	Content as % of control	Rate of loss %/hr.
10	5 hr. 9 min.	51.2	82	3.40
11	5 " 1 "	25.8	97	0.52
12	5 " 8 "	29.0	87	2.43
13	5 " 0 "	19.2	78	4.42
14	5 " 8 "	26.6	86	2.66

*Cyclopropane.* The results of the experiments with cyclopropane are shown in Table 3. The rate at which adrenaline was lost from the suprarenal glands varied between 0.65%/hr. and 10.5%/hr., a difference which is outside the range of experimental error. We were unable to account for this variation by the factors under observation; the average concentration of cyclopropane given to each cat varied only between 19.9 and 23.7% and the average blood pressure between 98 and 114 mm. The effect evidently arises from the different reaction of each animal to the anaesthetic.

TABLE 3. The effect of cyclopropane anaesthesia on the adrenaline content of the innervated suprarenal

Cat no.	Period of anaesthesia	Average cyclopropane concentration %	Average blood pressure mm. Hg	Content as % of control	Rate of loss %/hr.
15	5 hr. 10 min.	19.9	114	65	0.76
16	5 " 1 "	21.1	110	77	4.57
17	5 " 0 "	20.9	101	47	10.58
18	5 " 3 "	21.8	114	96	0.65
19	4 " 55 "	23.7	98	95	1.15

In Fig. 2 the average rates of loss of adrenaline under the three types of anaesthesia are compared. Under ether it is greatest, being  $6.7 \pm 1.4\%$ /hr. and it is least under pentobarbitone, being only  $2.7 \pm 0.6\%$ /hr. The average

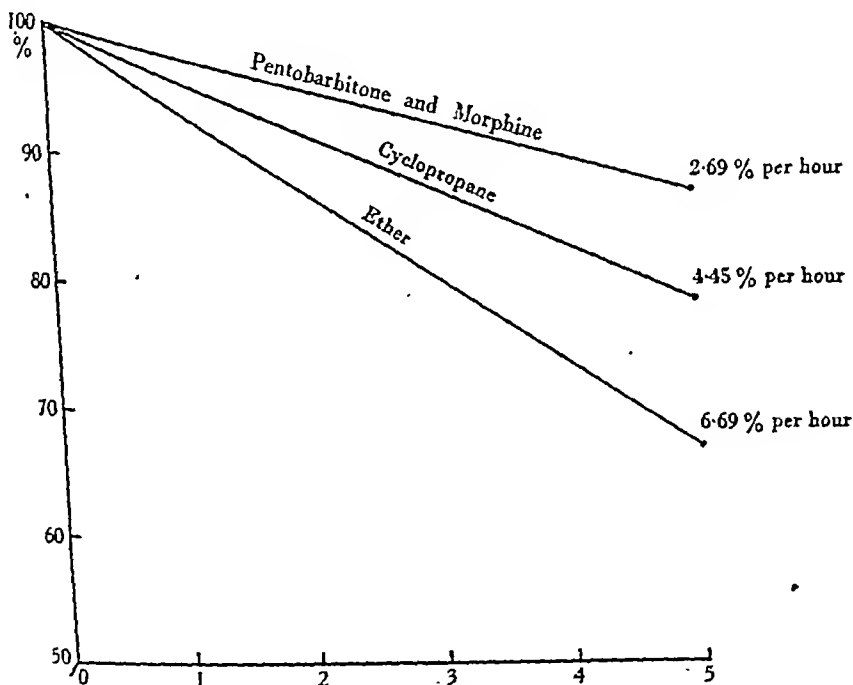


Fig. 2. A comparison of the rate of adrenaline loss from the innervated suprarenal glands of cats under different anaesthetics. Ordinate: adrenaline content as percentage of that of the denervated glands. Abscissa: duration of the anaesthesia in hours.

rate of loss under cyclopropane is  $4.45 \pm 1.8\%$ /hr. The highest rate of secretion in any animal was under ether ( $13.5\%$ /hr.) and the lowest under pentobarbitone ( $0.52\%$ /hr., not significant).

## DISCUSSION

The adrenaline content in the suprarenal glands depends both upon the rate of synthesis and the rate of secretion. Thus only if the rate of secretion exceeds the rate of synthesis does a loss of adrenaline from the gland occur. In our experiments we measured this loss, not absolutely, but in relation to the estimated original content of the gland.

The quantities of adrenaline secreted must have been greater than the figures indicate. Nevertheless the blood pressure during anaesthesia was not raised and indeed when ether was given the blood pressure gradually fell. We believe that the action of ether on the medulla causes an increase in the sympathetic outflow. Cattell [1923] and Bhatia & Burn [1933] have shown that this produces a vaso-constriction. At the same time adrenaline is being secreted, and it is well known that small doses of adrenaline can produce vaso-dilatation during ether anaesthesia [Macdonald & Schlapp, 1926]. This may explain the finding of Herrick, Essex & Baldes [1932] that ether increases the blood flow through the hind limb of the dog. The gradual fall of blood pressure we observed during deep ether anaesthesia may, therefore, be due to two factors: first the vaso-dilator action of the adrenaline, and secondly the direct action of ether on the heart.

Under cyclopropane anaesthesia the blood pressure was found to be normal in spite of a wide variation in the adrenaline loss from the suprarenal gland. Meek, Hathaway & Orth [1937] showed that, under cyclopropane anaesthesia, injected adrenaline readily produces ventricular tachycardia and fibrillation in dogs. This has been attributed to an increased excitability of the heart muscle accompanied by an increase in vagal tone. This indicates that the vaso-motor control is increased by cyclopropane and compensates for any adrenaline secretion occurring; the blood pressure remains normal.

## SUMMARY

A loss of adrenaline from the suprarenal glands of cats was observed during anaesthesia.

Under ether  $6.7 \pm 1.4\%$  (6) of the original content was lost per hr., under cyclopropane  $4.45 \pm 1.8\%$  (5) and under pentobarbitone-morphine  $2.7 \pm 0.6\%$  (5).

We wish to express our gratitude to Prof. J. H. Burn for his help and advice, and to the Nuffield Department of Anaesthetics for the loan of apparatus.



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THE EFFECT OF VARIATIONS IN THE SUBARACHNOID  
PRESSURE ON THE VENOUS PRESSURE IN THE  
SUPERIOR LONGITUDINAL SINUS AND  
IN THE TORCULAR OF THE DOG

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Although Weed & Flexner [1933] have confirmed the earlier findings of Becht [1920] that the venous pressure in the torcular is uninfluenced by variations in the subarachnoid pressure, Wright [1938] has found that changes in venous pressure can readily be brought about by varying the subarachnoid pressure. He measured the venous pressure in the posterior part of the superior longitudinal sinus. A slight rise in the venous pressure was observed when the subarachnoid pressure was first raised; this, however, fell rapidly to a low level and remained there as long as the raised subarachnoid pressure was maintained. In another series of experiments, Wright [1938] studied the effect of a gradual rise in subarachnoid pressure on the rate of blood flow through the superior longitudinal sinus. A decrease in blood flow was observed when the pressure was first raised with a progressive reduction until complete cessation occurred, when the subarachnoid pressure exceeded the systolic arterial pressure. A study has been made in the following experiments of the effect of variations in subarachnoid pressure on the venous pressure in the superior longitudinal sinus and in the torcular of the dog.

EXPERIMENTAL PROCEDURE

Difficulty in obtaining dogs owing to the war conditions has made little selection possible; diseased and grossly undernourished animals, however, were always rejected. The subarachnoid pressure was measured with a needle in the cisterna magna. Variations in pressure were effected and maintained in the manner already described in earlier publications [Bedford, 1935, 1936]. The highest pressure employed in these experiments was 500 mm. normal saline solution and the lowest 0 mm. normal saline solution. The venous pressure in the superior longitudinal sinus was recorded in the following way.

A cut was made with a  $\frac{1}{4}$  in. trephine through the bone over the posterior part of the sinus and continued down to stop short of the inner table. The disc of bone contained in the trephine could usually be broken away, leaving the sinus covered by the inner table and a variable amount of relatively soft diploë. The bone was then picked away with sharp forceps so as to expose a portion of sinus large enough to admit the recording needle. The sinus could usually be recognized by its dark colour. The recording needle (gauge 18) was introduced so as to point towards but not to enter the torcular. The needle was connected with a manometer of 1 mm. bore containing a 4% solution of sodium citrate. A device was also provided by which the needle could, when necessary, be flushed with citrate solution.

A different technique was used to record the venous pressure in the torcular. The torcular in the dog is not an intracranial structure; it lies, like the lateral sinuses, in the substance of the bone. It is possible, therefore, to measure the pressure of the blood in its interior without opening the cranium. This was effected in the following way. A dental bur was first selected capable of making a hole through the skull bone which would just admit a gauge 18 needle and leave no space by which fluid could escape along the side of the needle. A hole was then bored through the external occipital protuberance directly into the torcular. Care was necessary to prevent the bur from striking the thin anterior wall of the torcular, which is easily perforated. The bur was immediately withdrawn after the torcular had been entered and the needle introduced. A close fit ensued if care had been taken in the choice of bur. The needle was connected with the recording manometer in the manner already described. The experiments were performed with the animals in the horizontal position.

The animals were anaesthetized with intratracheal ether administered by a pump in all but two experiments when amytal (Lilly) was the anaesthetic. The amytal, in a proportion of 0.08 g. per kg. of body weight and in a 5% solution in water, was injected into the peritoneal cavity. The animals were then kept quiet in a darkened room for 20 min. when they were found to be in a satisfactory state of anaesthesia. The animals were killed at the end of the experiments and a careful examination made of the superior longitudinal sinus, the torcular and the lateral sinus for the presence of ante-mortem clot.

## RESULTS

*The effect of variations in subarachnoid pressure on the venous pressure in the membranous superior longitudinal sinus.* A rapid increase in subarachnoid pressure from the pressure level of the cerebrospinal fluid at the beginning of the experiment to 500 mm. normal saline solution was invariably accompanied by a fall in venous pressure (Fig. 1). The extent of the fall was roughly proportional to the rate at which the subarachnoid pressure was increased. If 1 min. was spent in raising the subarachnoid pressure to 500 mm. normal saline

solution, a fall in venous pressure which averaged 20 mm. normal saline solution was observed. When the subarachnoid pressure was raised more slowly and 1 min. allowed for each increase in 100 mm. normal saline solution at intervals of 3 min., no change was observed in the venous pressure in a quarter of the animals while a fall of pressure which averaged 10 mm. occurred in the remainder. When the subarachnoid pressure was raised still more slowly, a slightly greater proportion of animals presented no change in venous pressure.

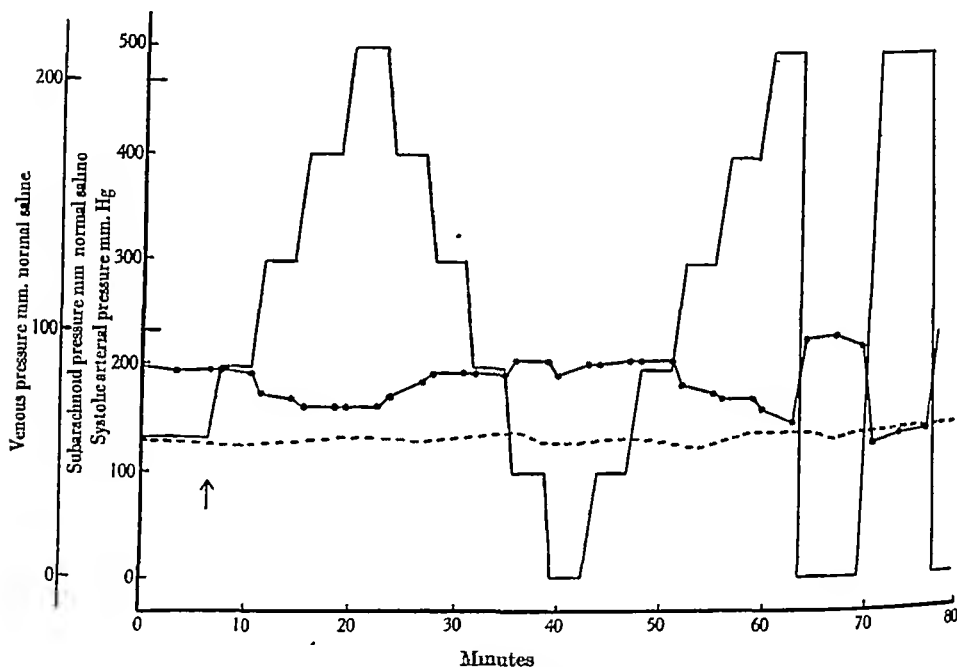


Fig. 1. The effect of variations in subarachnoid pressure on the venous pressure in the superior longitudinal sinus. — Subarachnoid pressure. —●—●— Venous pressure. .... Systolic pressure in femoral artery. The arrow indicates the point at which the subarachnoid pressure was artificially varied. Ether anaesthesia.

It was found, however, that the animals which presented no change in venous pressure when the subarachnoid pressure was first raised, frequently did so after it had been raised and lowered for the second or third occasion; thereafter a rise in subarachnoid pressure was accompanied by a fall in venous pressure. Lowering the subarachnoid pressure from 500 mm. normal saline solution to its original level was accompanied by corresponding changes in the opposite direction and the venous pressure assumed its original level. Occasionally, however, a level slightly higher than the original was assumed but this rarely persisted and the original level was generally regained within 15 min. The

extent of response of the venous pressure in any particular animal remained fairly constant on each subsequent elevation of pressure. Except for minor variations, the venous pressure level assumed on raising the subarachnoid pressure was generally maintained (Fig. 2); in a few instances, however, the pressure rose gradually until the original level was regained after 5-20 min.

The above changes in venous pressure occurred independently of variations in systemic blood pressure; the systemic arterial blood pressure was uninfluenced by changes in subarachnoid pressure of the magnitude employed in

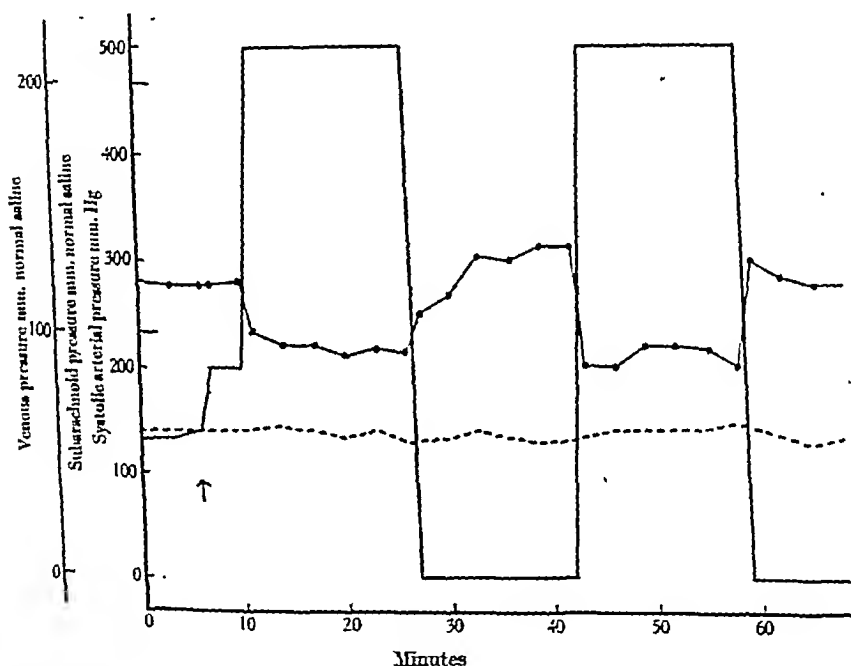


Fig. 2. The effect of prolonged variations in subarachnoid pressure on the venous pressure in the torcular. Constants as in Fig. 1. Ether anaesthesia.

these experiments. The results obtained in two experiments in which the animals were anaesthetized with amytal were similar to those in which the animals were anaesthetized with ether. Experiments were performed on seventeen dogs.

*The effect of variations in subarachnoid pressure on the venous pressure in the torcular.* The effects of variations in subarachnoid pressure on the venous pressure in the torcular were identical with those observed when the venous pressure was recorded in the posterior part of the membranous superior longitudinal sinus. Experiments were performed on six dogs.

## DISCUSSION

Although the results of the above experiments fail to confirm the finding of Weed & Flexner [1933] that the venous pressure in the torcular is uninfluenced by changes in subarachnoid pressure, they do, however, provide evidence for the existence of a compensating mechanism by which the cerebral circulation adapts itself to the changes in subarachnoid pressure. The efficiency of this mechanism varies considerably in different animals. In some instances compensation appeared to be complete and the venous pressure remained uninfluenced by changes in subarachnoid pressure. Nearly every animal, however, which presented no change in venous pressure when the subarachnoid pressure was first raised, showed a fall after the subarachnoid pressure had been raised for the second or third time.

Wright [1938] has provided strong experimental evidence that no compression or collapse of the superior longitudinal sinus occurs when the subarachnoid pressure is increased to levels even greater than the systolic arterial blood pressure; it is evident, therefore, that in the absence of variations in the systemic circulation, any change observed in the sinus is a direct consequence of variations in the cerebral circulation. A fall in venous pressure invariably followed a sudden rise in subarachnoid pressure, and the new venous pressure level generally persisted as long as the raised subarachnoid pressure was maintained. It was also found that, on lowering the subarachnoid pressure, a reduced venous pressure frequently accompanied a lower subarachnoid pressure level than was observed when the pressure was increased. These findings are probably related to Wright's observation that cerebral vessels which have been collapsed as a result of raising the subarachnoid pressure require a lower pressure level for the reversal of the process than that at which they first collapsed. Wright [1938] considers the phenomenon is in conformity with the law that resistance to the passage of fluid through a tube varies inversely as the fourth power of the radius. The diameter of the collapsed vessels is negligible at the beginning of filling, and as no active expansion can take place, the resistance to filling is considerable.

Becht [1920] measured the venous pressure by screwing a brass tube 4 mm. in diameter into a drill hole made directly into the torcular through the external occipital protuberance. As might be anticipated, considerable difficulty was encountered from clotting and this method of recording the venous pressure was abandoned by Weed & Hughson [1921] who exposed the superior longitudinal sinus over the posterior third of its course by carefully removing the bone in a sagittal groove by means of a rongeur. A shortened lumbar puncture needle was then introduced into the sinus and passed backwards into the torcular. This technique appears to have been employed by Weed and his co-workers without modification throughout their experiments. It is open,

however, to the grave objection that the cranium can be no longer regarded as a closed chamber after the sinus has been exposed. Nevertheless, it has been shown in the present series of experiments that if care be taken to expose a portion of the sinus sufficient only to admit the recording needle, the results are identical with those obtained when the venous pressure is recorded directly from the torcular.

No attempt has been made in this investigation to study the effect of subarachnoid pressures greater than 500 mm. normal saline solution; pressures of greater magnitude than this were considered to depart too far from the physiological. Weed & Flexner [1933] also restricted themselves mainly to the study of the effect of pressure variations up to 450 mm. normal saline solution, although Wright [1933], at times, used pressures which even exceeded the systolic arterial pressure.

Ethër was the anaesthetic used by Becht [1920] and by Weed & Flexner [1933] throughout their experiments and, except for two experiments in which intraperitoneal amytal was the anaesthetic, all the remaining experiments in the present series were performed on animals anaesthetized with intratracheal ether. It is noteworthy that similar results were obtained whether ether or amytal was the anaesthetic.

Weed & Flexner [1933] state emphatically that even profound changes in subarachnoid pressure are unaccompanied by changes in venous pressure in the superior longitudinal sinus. In a later paper, however, by Mortenson & Weed [1934] on 'The absorption of isotonic fluids from the subarachnoid space' a figure (Fig. 3, p. 463) is presented illustrating an experiment in which a study was made of the effect of raised subarachnoid pressure on the venous pressure in the superior longitudinal sinus and on the rate of inflow of normal saline solution into the subarachnoid space of the dog. The subarachnoid pressure was raised after an initial control period of 30 min. in multiples of the normal pressure and at intervals of 15 min. It is significant that a fall in venous pressure of some 20 mm. normal saline solution accompanied the raising of the subarachnoid pressure from 360 to 540 mm. normal saline. The reduced venous pressure level was maintained with slight variation for 15 min. when, on increasing the subarachnoid pressure to 720 mm. normal saline, it showed a tendency to assume a slightly lower level. Although no comment is made by Mortenson & Weed on the fall in venous pressure, it would appear to be in harmony with the findings in the experiments now under consideration.

To conclude, it would appear that although the cerebral circulation possesses considerable power of adaptation to changes in subarachnoid pressure, a rise in subarachnoid pressure from the normal cerebrospinal fluid pressure level of approximately 120-500 mm. normal saline solution, is accompanied in the majority of animals by a slight but persistent fall in the venous pressure in the sinus and in the torcular.

## SUMMARY

1. A study has been made of the effect of variations in subarachnoid pressure on the venous pressure in the membranous superior longitudinal sinus and in the torcular of the dog.

2. A rapid increase in subarachnoid pressure from the pressure level of the cerebrospinal fluid to 500 mm. normal saline solution was invariably accompanied by a fall in venous pressure which averaged 20 mm. normal saline solution. Identical results were obtained whether the venous pressure was recorded in the membranous superior longitudinal sinus or in the torcular.

3. When the subarachnoid pressure was raised more slowly and 1 min. allowed for each increase of 100 mm. normal saline solution at intervals of 3 min., a fall in venous pressure which averaged 10 mm. normal saline solution was observed in the majority of the animals.

4. The fall in venous pressure generally persisted as long as the raised subarachnoid pressure was maintained; the original level was, however, regained when the subarachnoid pressure was lowered.

5. It is concluded that, although the cerebral circulation possesses considerable power of adaptation to changes in subarachnoid pressure, a rise in subarachnoid pressure from the pressure level of the cerebrospinal fluid to 500 mm. normal saline solution even when effected slowly, is generally accompanied by a small but persistent fall in the venous pressure in the superior longitudinal sinus and in the torcular.

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## ACCELERATION OF THE HEART BY THE VAGUS IN CATS AFTER COMPLETE SYMPATHECTOMY

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In the course of experiments on totally sympathectomized cats, we had occasion to observe the effects on the heart of stimulation, after the administration of atropine, of the peripheral ends of the vagus nerves cut in the neck.

In the normal cat, stimulation of the vagus after atropine does not affect the heart frequency, but in some of the sympathectomized cats we have observed accelerator effects. The existence of accelerator fibres has been demonstrated in the dog's vagus [Morgan & Goland, 1932], but there appears to be general agreement that the vagus of the cat does not contain such fibres [Hering, 1924], and our observations on normal cats confirmed this. We have therefore undertaken some experiments to explain this anomalous acceleration which we observed in the sympathectomized animals.

### RESULTS

Those cats in which we observed the abnormal acceleration by the vagus had, in all cases, been subjected to complete sympathectomy by the method of Cannon, Newton, Bright, Menken & Moore [1929], with the slight modification that, in completing the operation at the upper end of each thoracic chain, we gripped the stellate ganglia with forceps, and removed them by forcible avulsion, breaking the rami communicantes, the nervi accelerantes and the two branches of the annulus Vieussensii near their junction below the middle cervical sympathetic ganglion, leaving the latter in situ. Two to two and a half months had in all cases elapsed between the first stage of the operation and the final experiment.

In one of our experiments on a sympathectomized cat, after decerebration, we observed that the normal fall of blood pressure produced by stimulation of the peripheral end of the cut right vagus nerve in the neck was followed, when the stimulation was ended, by a rise of blood pressure and an acceleration of the heart. The administration of atropine in a dose sufficient to abolish

the depressor action of the vagus, then revealed the full extent of this acceleration (Fig. 1). The heart rate before stimulation was 144 and, at the peak

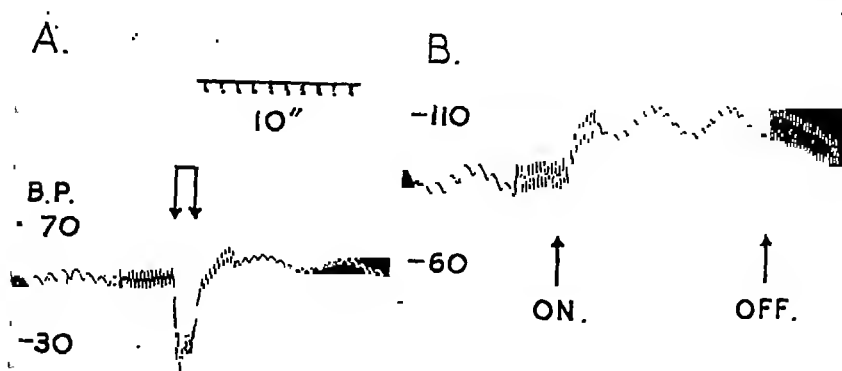


Fig. 1. Cat after total sympathectomy, record of arterial blood pressure. Stimulation of peripheral end of right vagus. A before, and B after 0.4 mg. atropine. Before and after the first stimulation, and before and during the second, the recording surface has been accelerated to show the heart frequency.

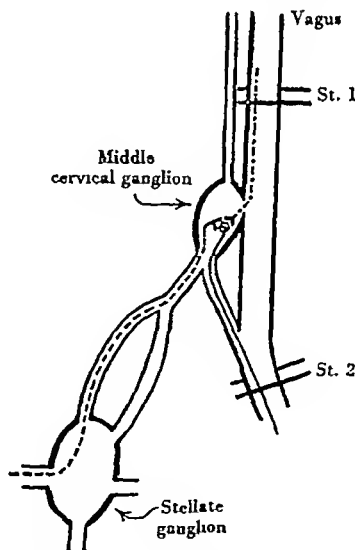


Fig. 2. Diagram showing probable path of abnormal accelerator fibres through middle cervical sympathetic ganglion. — normal fibres. - - - - - degenerated fibres. - - - - - vagus fibres forming synapses in the cervical sympathetic ganglion. St. 1 and St. 2 show sites of stimulation; see text.

of the quickening, 240 per min., an increase of 66%. Stimulation of the left vagus had a similar, but less conspicuous, effect, the maximum quickening being about 43%.

The cardio-acceleration had, in this experiment, a latency of 1-2 sec., indicating that it was probably due to a direct, nervous effect upon the pacemaker. It was necessary, however, to rule out the possibility of an effect on the heart, sensitized by partial sympathetic denervation, of cardio-accelerator substances liberated in the periphery from adrenergic nerve

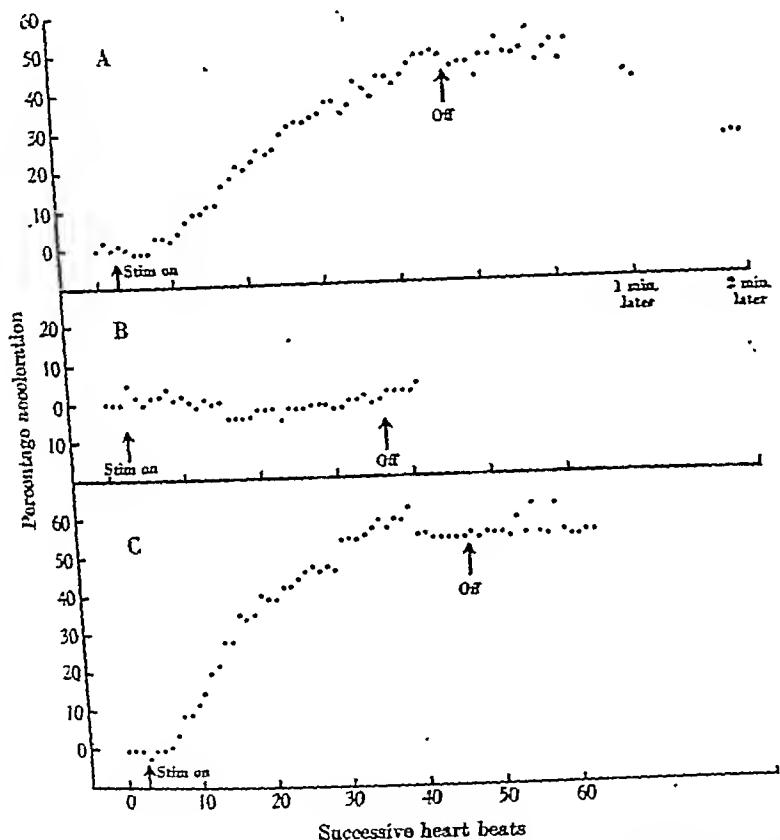


Fig. 3. Acceleration of the heart produced by stimulating the vagus before and after the application of nicotine to the middle cervical sympathetic ganglion. A, stimulation of vagus above middle ganglion, before nicotine. B, after nicotine. C, stimulation below the middle ganglion after nicotine.

endings excited through the vagus. We consequently crushed the oesophagus in the abdomen immediately below the diaphragm in such a way as to interrupt the continuity of the abdominal strands of the vagus. This procedure did not alter the accelerator effect of stimulation of the vagus. Similarly, an abdominal evisceration, including the kidneys, did not change the response. We felt reasonably confident, therefore, that the effect was due to a direct action of accelerator fibres reaching the heart from the vagus. Similar, but smaller,

accelerator actions were seen in other sympathectomized animals, viz. with stimulation of the right vagus 50 and 43 %, and with stimulation of the left vagus 26, 22 and 20 %.

*Nature and origin of the accelerator fibres.* The fibres to which the stimulation was applied were certainly in the vagus, which was dissected cleanly from the cervical sympathetic trunk. If they were preganglionic fibres, they might form connexions with nerve cells in the heart or in the middle cervical ganglion, which is closely attached to the vagus, as shown in Fig. 2. We put the latter suggestion to the test by painting nicotine on the middle cervical ganglion, and found it to be correct. Thus, in one experiment, in which the heart frequency was recorded electrocardiographically, stimulation of the vagus on the cephalic side of the ganglion (*St. 1*, Fig. 2) had produced, after atropine, a 50 % acceleration of the heart. We then painted the ganglion with 2 % nicotine, which caused a transient acceleration. When the rate had again become constant, we repeated the stimulation of the vagus, which was now without perceptible effect. The electrodes were now applied to the vagus below the ganglion, distal to the junction with it of the twig from the ganglion, known to carry postganglionic sympathetic fibres to the heart. Stimulation at this point (*St. 2*, Fig. 2) now produced a 60 % acceleration (Fig. 3). The persistence of the nicotine paralysis was verified by renewed stimulation above the ganglion, which was still without effect.

The probable connexion with removal of the upper end of the thoracic chain, and particularly of the stellate ganglion, being obvious, we attempted to produce the condition deliberately by operations limited to those ganglia. Removal of the stellate ganglia and the next three thoracic ganglia was carried out in two cats, and section of the white rami of the stellate ganglia in two others, the tests for accelerator vagus function being made between 3 and 3½ months later. In no case, however, was the accelerator effect observed as a sequel to these more limited operations.

## DISCUSSION

We were long puzzled to account for this abnormal vagus action, occurring in several of a small series of totally sympathectomized cats, but not in any of those in which the operation was limited to the upper thoracic ganglia. We considered the possibility that the heart, deprived of its main sympathetic supply by removal of the stellate ganglia, might have been rendered sensitive thereby to the action of a normally ineffective adrenergic component in the cat's vagus. The normal dog's vagus demonstrably contains accelerator fibres, which are presumably adrenergic. If the effect were of this kind, however, simple removal of the stellate ganglion, or mere section of its accelerator branches must have been equally effective in eliciting it, and it should have

appeared in a few days after the operation. On the other hand, we have shown that the cardiac acceleration was not due to the action of cardio-accelerator substances liberated peripherally by adrenergic vagal fibres, since it was still evoked by stimulation of the vagus after crushing the oesophagus in the abdomen, or removal of all the abdominal viscera. We then considered the possibility that preganglionic denervation of the cells of the middle cervical ganglion had been followed by the formation of new synaptic connexions with them by the fibres of the closely associated vagus nerve. Postganglionic fibres from the cells of the ganglion produce acceleration of the heart [Langley, 1892; Cannon, Lewis & Britton, 1926], and preganglionic vagus fibres, like other cholinergic fibres, would form functional synaptic connexions with them if the conditions for such regeneration were provided. The preganglionic denervation, however, would be equally well produced by removal of the stellate ganglion, or by mere section of its white rami, or of the limbs of the annulus, and the vagus did not acquire the accelerator function after any of these procedures. Considering how complete sympathectomy could favour the formation of new junctions with cells of the middle cervical ganglion, we believe that we have found the explanation, not in the greater extent of the removal, but in the more forcible avulsion of the stellate ganglion which a speedy conduct of the larger operation led us to employ. Cajal [1928] has shown that, although the presence of degenerating fibres exercises a powerful attraction to the ingrowth of regenerating fibres from neighbouring nerves which have been injured, fibres which are normal and intact never contribute to such regeneration. A very small degree of injury, however, leads to the sprouting of branches, which can then find their way into the track of degenerating fibres. If the stellate ganglion is roughly dragged from its bed, severe traction is exerted on the middle cervical ganglion and on the vagus nerve closely attached to it, before the branches of the annulus break. With such a procedure it is probable that some vagus fibres might be sufficiently injured to form regenerative branches, while, on the other hand, the conditions for such injury would be uncertain and irregular, as the acquisition of accelerator function by the vagus was found to be. This seems to us to be the most probable explanation of the effect which we observed, inasmuch as it has appeared only in some of our sympathectomized cats, and in none in which the middle cervical ganglion was denervated by methods other than avulsion of the stellate ganglion. If we are right, it must be regarded as an accident of operative procedure, without any general physiological importance. In normal times we might have put this idea to a more direct test, by removing the stellate ganglion only, by avulsion, in a sufficient series of cats, and waiting over the requisite period of months to discover whether the vagus accelerator effect appeared in any. The intervention of the war, however, diverted our activities into other directions. We think it desirable to place

our experience on record, as evidence of the care which must be taken, both in the performance of operations likely to cause damage and consequent regrowth of nerve fibres, and in the interpretation of results after such procedures.

#### SUMMARY

1. Stimulation of the peripheral end of the vagus nerve, cut in the neck, may cause in sympathectomized cats an acceleration of the heart.
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# PULMONARY CIRCULATION TIMES BEFORE AND AFTER FUNCTIONAL CLOSURE OF THE DUCTUS ARTERIOSUS

By A. E. BARCLAY, J. BARCROFT, D. H. BARRON, K. J. FRANKLIN  
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(Received 3 July 1942)

Since 1937 we have studied by means of direct and indirect X-ray cinematography [Barclay, Franklin & Prichard, 1940] the circulation in the foetal lamb delivered by Caesarean section. We have already detailed many of the results [e.g. Barclay, Barcroft, Barron & Franklin, 1939; Barclay, Barcroft, Barron, Franklin & Prichard, 1941], but we have not previously published one particular series of findings, namely the pulmonary circulation times before and after functional closure of the ductus arteriosus. These are of interest for two reasons, the first being their absolute smallness even before closure of the ductus, the second being their reduction after its closure.

The records from which we have extracted the times have all been provided by direct X-ray cinematography, and recording has been at the rate of 178 or 200 frames per min. On examining the films, we found that we could determine thirty-seven pulmonary circulation times in the records of thirteen foetuses. We had to decide in each instance which frames showed comparable densities of shadow produced by injected contrast medium at the beginnings of the pulmonary arteries and the endings of the pulmonary veins. This is obviously a matter of individual judgement, but we believe that our experience of such work and the number of instances have eliminated any serious source of error.

Among the records are a few from foetuses of about 100 days (full term is about 147 days). It is of interest to note that the circulation times in these were of the same order as in the other, mature foetuses. Different anaesthetics were used, but this variable, again, seemed to be without any appreciable effect. On the other hand in any particular foetus the circulation time invariably fell if the ductus arteriosus closed and invariably rose again if it subsequently reopened. The average time with the ductus wide open was 2.7 sec., the range from 2 to 6 sec. The corresponding figures with the ductus closed were 1.4 sec., and 1.3-3 sec. The average with the ductus incompletely closed was intermediate, namely, 1.6 sec.

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## SUMMARY

In a series of foetal lambs delivered by Caesarean section functional closure of the ductus arteriosus was followed by a reduction in the average pulmonary circulation time from 2.7 to 1.4 sec.

## REFERENCES

- Barclay, A. E., Barcroft, J., Barron, D. H. & Franklin, K. J. [1939]. *Brit. J. Radiol.* 12, 505.  
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Barclay, A. E., Franklin, K. J. & Prichard, M. M. L. [1941]. *Brit. J. Radiol.* 14, 105.

As examples we may reproduce in full the details regarding three practically full-term foetuses, in one of which the ductus never fully closed, in the second of which it closed and later reopened, as happens if the animal deteriorates, and in the third of which it closed and remained closed for the duration of the experiment.

TABLE 1

Serial no. of foetus	Time from delivery	Time from ligation of cord	Ductus arteriosus	Time of pulmonary circulation in sec.
S 22	20 sec.	20 sec.	Open	3.0
	5 min.	5 min.	Open	3.7
	10 "	10 "	Open	3.0
	20 "	20 "	Open	3.0
	30 "	30 "	? Partly closed	2.2-3
S 27	9 "	9 "	Open	2.3
	20 "	20 "	Partly closed	1.7
	32 "	32 "	Closed	1.0
	41 "	41 "	Closed	1.0
	55 "	55 "	? Slightly open	1.3
	80 "	80 "	Open	2.3
S 30	6 "	—	Open	>2.0*
	11 "	4 "	Closed	1.7
	17 "	10 "	Closed	1.7
	26 "	19 "	Closed	1.3
	36 "	29 "	Closed	1.0

\* This shot did not last for more than 2 sec., so we cannot give the exact circuit time. Actually we have only one time for the pulmonary circuit in a mature foetus before ligation of the cord. This was 3.3 sec.

As we have said above, the halving of the pulmonary circulation time through closure of the ductus is not the only point of interest in our findings, for the absolute values themselves are far smaller than one would anticipate from statements in text-books and elsewhere. For example, one such authority gives the average pulmonary circulation time in Man as 11 sec., and the range as 5-17 sec. Robb & Steinberg [1940, Table 2], on the other hand, by radiographic procedures not dissimilar in principle to our own, found much shorter times in Man. The contrast medium, injected into an arm vein, rendered the superior vena cava and right atrium radio-opaque in 1.5 sec., the right ventricle and pulmonary arteries in 2-3 sec., and the pulmonary veins and left atrium in 5-7 sec. So the pulmonary circulation time was between 2 and 5 sec., a figure comparable with the 1.4 sec. in our foetuses in view of the much shorter distance to be traversed in these latter.

Apart from pulmonary circulation times we have been able to obtain from our records eight times for the circuit from the brachiocephalic artery to the anterior vena cava. The main interest of these is that they show a decrease from over 10 or 11 sec. at about 100 days to something in the neighbourhood of 5 sec. at 140 days. If future findings tell a similar story, it will be worth while to investigate the cause and significance of such a decrease.

## SUMMARY

In a series of foetal lambs delivered by Caesarean section functional closure of the ductus arteriosus was followed by a reduction in the average pulmonary circulation time from 2.7 to 1.4 sec.

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# THE CIRCULATORY EFFECTS OF SOME ISO-THIOUREA DERIVATIVES, WITH SPECIAL REFERENCE TO THE SENSITIZATION OF ANIMALS TO THE PRESSOR ACTION OF ADRENALINE

BY F. N. FASTIER AND F. H. SMIRK

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*(Received 27 July 1942)*

Following the discovery of its action on the blood pressure [Smirk, 1941], S-methyl iso-thiourea sulphate has been used clinically by one of us (F. H. S.) as a pressor agent and some of its pharmacological properties have already been described [McGeorge, Sherif & Smirk, 1942]. While these investigations were in progress, a rapid examination of some eighty thiourea and iso-thiourea derivatives was carried out in the hope that other substances of therapeutic value might be obtained. Several iso-thiourea derivatives, especially those without N-substituents, were found to resemble S-methyl iso-thiourea in that their intravenous injection produced one or more of the following effects: (1) a prompt rise of arterial pressure, which with several derivatives remained elevated for periods ranging from five minutes to an hour or more; (2) slowing of the heart beat; (3) increased respiratory movements; (4) an increase in and sometimes prolongation of the pressor action of adrenaline. This paper records our observations on the circulatory properties of those iso-thioureas which were chosen for further study, viz. S-methyl, S-ethyl, S-iso-propyl, S-n-butyl and S-tert.-amyl iso-thioureas, of general formula  $R.S.C(:NH)NH_2$ , where R is an alkyl group.

thorax. The apparatus used for perfusing the hindquarters of the rat is illustrated in Fig. 1, and allows the alternate use of two perfusion fluids. A rat, of 150 g. or more, is killed by a blow on the head. The abdomen is widely opened and a cannula is introduced downwards into the abdominal aorta, a little above its bifurcation and below its main visceral branches. The rat is now bisected transversely above the level of the cannula, and the preparation of hindquarters is pithed by means of a needle. The cannula is now attached to the perfusion apparatus, the weight of the preparation being carried by clips attached to the skin. With the preparation in position the lower limbs of the rat are severed at the lower ends of the tibiae, and the tail is cut short about an inch from its insertion. These amputations provide a free exit for the perfusion fluid from severed arteries and little or no visible oedema develops. In order to facilitate the injection of small quantities of drugs into the rubber tube immediately before the cannula, the latter is held firmly by a clamp so that the preparation is not disturbed mechanically by the injection. The tuberculin syringes employed for injection were fitted with a clock spring pressing on to the side of the plunger so as to prevent this being thrust backwards by the head of pressure. The effect of *iso*-thioureas upon the alimentary canal was studied by means of isolated rabbit or rat intestine suspended in oxygenated Ringer-Locke or Ringer-Tyrode solution at 39° C., in an organ bath similar to that described by Burn & Dale [1922]. Excised spirals [Lewis & Koessler, 1927] from the femoral artery and aorta of the cat were also used in organ bath experiments. The compounds were

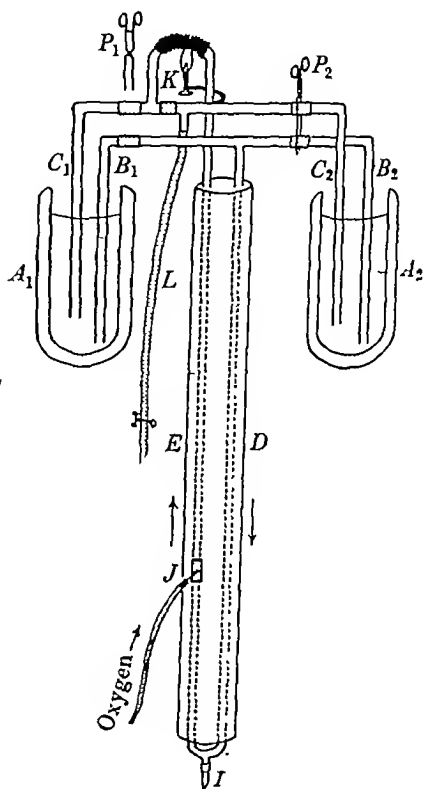


Fig. 1. Device for perfusion of isolated organs.  $A_1$ ,  $A_2$ , Dewar flasks containing the perfusion fluids.  $B_1$ ,  $B_2$ , tubes conducting the fluid to the perfusion cannula  $I$ .  $C_1$ ,  $C_2$ , return tubes. The tubes  $D$  and  $E$  are enclosed in asbestos packing surrounded by cardboard.  $K$ , flame heating wire gauze around the return tube from the cannula.  $J$ , hypodermic needle conducting oxygen from a pressure reservoir to the fluid in  $E$ , the oxygenation and circulation of the perfusion fluid being thus maintained.  $P_1$ ,  $P_2$ , clamps for changing from one perfusion fluid to the other. Opening the tube  $L$  for a few moments before and after changing from one perfusion fluid to the other prevents contamination of one fluid by the other.

prepared by the usual methods [Sprague & Johnson, 1936; Taylor, 1917], purity being confirmed by the melting point. No reference was found to either *S-n*-butyl iso-thiourea hydrobromide (m.p. 80° C.) or *S-tert.*-amyl iso-thiourea salicylate (m.p. 133° C.). Their composition was checked by analysis.

## RESULTS

*S*-ethyl iso-thiourea was tested on four dogs, three rabbits and one cat; *S*-iso-propyl iso-thiourea on four dogs and two cats; *S-n*-butyl iso-thiourea on three dogs and two cats. In addition, each of the compounds was tested on 6–10 occasions on animals which had previously received other drugs, essentially similar results being obtained.

### *Effect on the blood pressure*

As *S*-methyl iso-thiourea has been used clinically for its pressor activity, it is of interest that initial doses (of 0.002 g./kg. upwards) of *S*-ethyl or *S*-iso-propyl

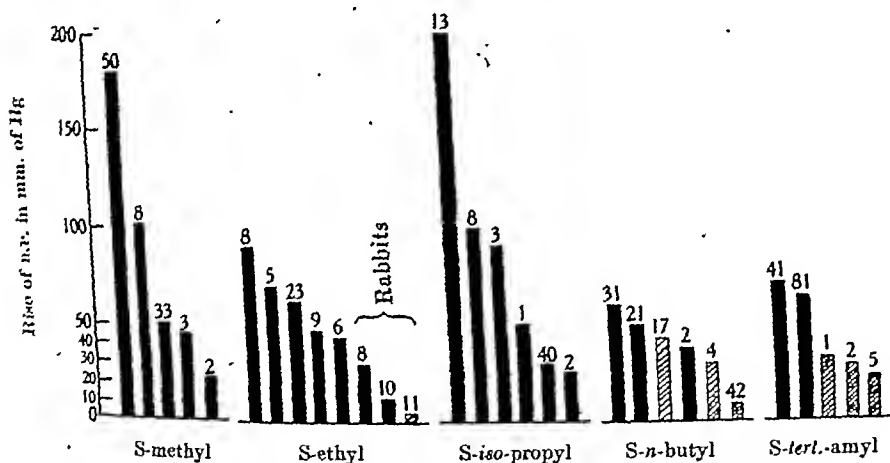


Fig. 2. Effect of iso-thiourea derivatives on the arterial blood pressure of cats and dogs. The dose (in mg./kg.) is given above each column. Each solid column represents a rise lasting at least 10 (and often more than 20) min. Each hatched column represents a temporary rise. Doses of *S-n*-butyl iso-thiourea usually produced a sharp fall of blood pressure either preceded by a transitory rise or followed by a prolonged rise except on three occasions (not shown) when only falls of blood pressure were observed. The results for *S*-methyl iso-thiourea are a representative selection from 30 to 40 experiments.

iso-thiourea have been found to give strong and well-maintained rises of blood pressure in all cats and dogs studied (Fig. 2). With the *S-n*-butyl and *S-tert.*-amyl derivatives, however, more variable results were obtained. While temporary rises were sometimes given by initial doses of *S-n*-butyl iso-thiourea, later doses produced only falls of blood pressure. Indeed, with all these compounds, when several injections were given in succession to the same

animal, the later injections had less effect, until ultimately little or no rise of blood pressure was obtained (Fig. 3). This phenomenon of tachyphylaxis was not exhibited by different *iso*-thioureas to the same degree. Our observations suggest that the other derivatives examined exhibit it to a greater extent than S-methyl *iso*-thiourea, and for this reason their pressor action is less likely to possess therapeutic value. Following several injections of S-ethyl *iso*-thiourea, a dose of S-methyl or S-*iso*-propyl *iso*-thiourea, which would be expected to produce a striking rise of blood pressure in a fresh animal, now has little

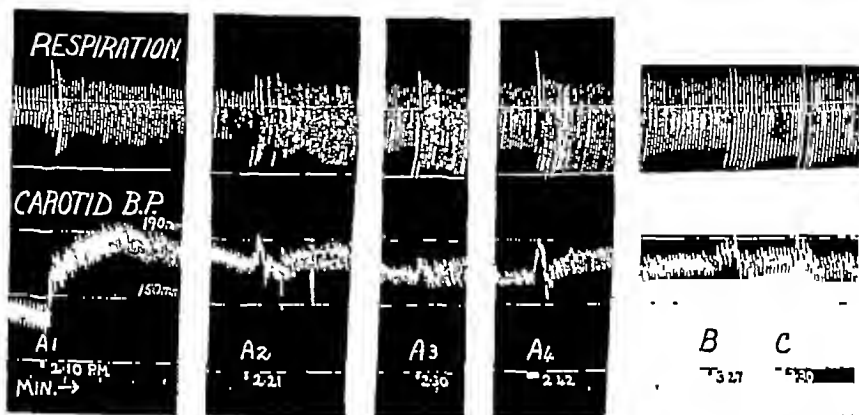


Fig. 3. Tachyphylaxis with repeated injections of *iso*-thiourea derivatives. 11 kg. dog. Sodium barbitone (2.3 g.). Vagi cut. S-ethyl *iso*-thiourea (each dose 0.1 g.) was given at  $A_1$ ,  $A_2$ ,  $A_3$ ,  $A_4$ ; S-methyl *iso*-thiourea (0.05 g.) at B; and S-*iso*-propyl *iso*-thiourea (0.1 g.) at C.

pressor effect (Fig. 3). Similar results were obtained after initial doses of S-methyl or S-*iso*-propyl *iso*-thiourea and the subsequent injection of S-ethyl *iso*-thiourea. Thus, after several doses of one of these substances, an animal is desensitized not only to the pressor action of the *iso*-thiourea used initially (tachyphylaxis) but also to that of other homologues.

#### *Effect on the blood vessels*

These experiments were carried out in order to see if the rise of blood pressure was brought about by peripheral vasoconstriction. Rats' hindquarters were pithed and the blood vessels perfused through the aorta with Ringer-Locke solution (see Fig. 1). It was found that doses of S-methyl and S-ethyl *iso*-thioureas (10–100  $\mu$ g.), dissolved in about 0.1 ml. of Ringer-Locke solution and injected into the perfusion cannula, reduced the rate of flow from the stump of a severed limb, apparently because of vasoconstriction (Fig. 4). Contraction of arterial spirals in Ringer-Locke solution by S-ethyl *iso*-thiourea was also demonstrated occasionally (Fig. 5), although the concentration required (1 in  $10^3$  to  $10^4$ ) was appreciably higher than that which brings about vasoconstriction in the whole animal (1 in  $10^5$ ).

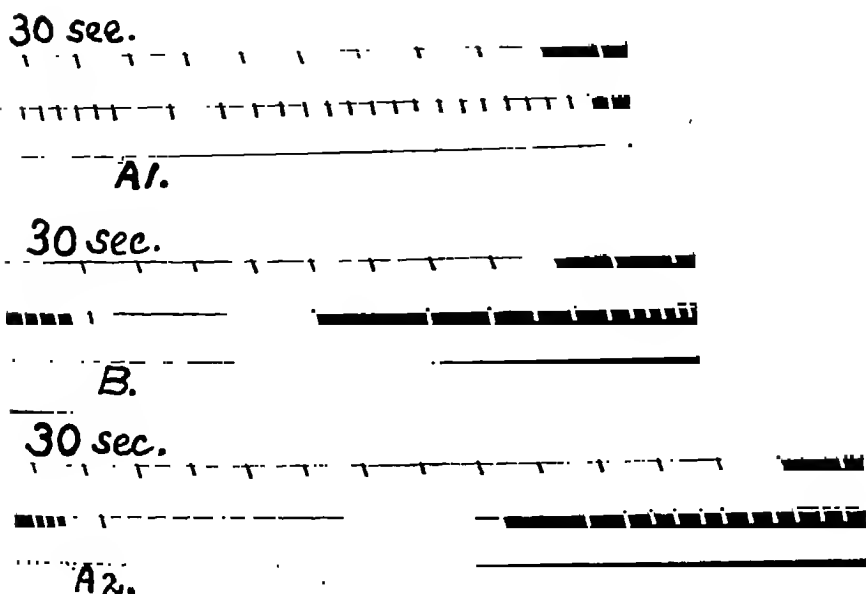


Fig. 4. Drop-counter record of perfusion of pithed hindquarters of rat (middle line) showing a decrease in the rate of drop formation in response to adrenaline ( $0.1 \mu\text{g.}$  at  $A_1$ ) and S-ethyl iso-thiourea ( $20 \mu\text{g.}$  at  $B$ ). Following injection of the latter, adrenaline ( $0.1 \mu\text{g.}$  at  $A_2$ ) has an enhanced effect.

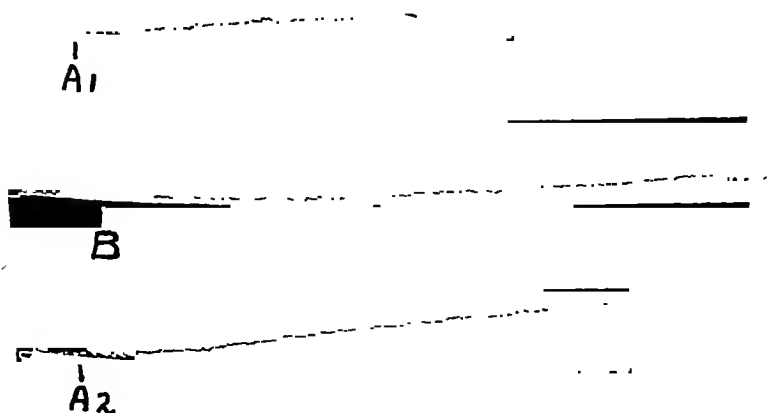


Fig. 5. Contractions of an arterial spiral (cat) produced by (i) adrenaline (4 parts in 10 million) at  $A_1$ , (ii) S-ethyl iso-thiourea (3 parts in 10 thousand) at  $B$  and (iii) adrenaline (4 parts in 10 million) at  $A_2$ . Sensitization to the vasoconstrictor action of adrenaline is indicated by the stronger contraction (at  $A_2$ ) following the administration of S-ethyl iso-thiourea.

*Sensitization to the pressor action of adrenaline*

When adrenaline is given before and after certain *iso*-thiourea derivatives, it is observed that its pressor effect has been enhanced by the *iso*-thiourea. This property has been studied more particularly with the compounds referred

TABLE 1. Sensitization to the pressor action of adrenaline by *iso*-thioureas

Substance	Degree to which the sensitivity to adrenaline was multiplied in individual experiments	Average
S-methyl <i>iso</i> -thiourea	2, 4, 4, 1, 2, 2	2.5
S-ethyl <i>iso</i> -thiourea	8, 4, 5, 8, 3 (2, 3, 2, in rabbits)	5.5
S- <i>iso</i> -propyl <i>iso</i> -thiourea	4, 1, 6, 4, 5, 7	4
S- <i>n</i> -butyl <i>iso</i> -thiourea	4, 2, 6, 5, 1, 1, 6, 4, 2	3
S- <i>tert</i> -amyl <i>iso</i> -thiourea	9, 8, 4, 10, 8	8

to in Table 1. Isolated observations indicate that some other homologues behave similarly. In these experiments on dogs and cats, the pressor response to several different doses of adrenaline (Parke Davis adrenalin chloride 1:1000 suitably diluted) was first determined. Following the subsequent administration of a 10 % aqueous solution of the substance under investigation (an

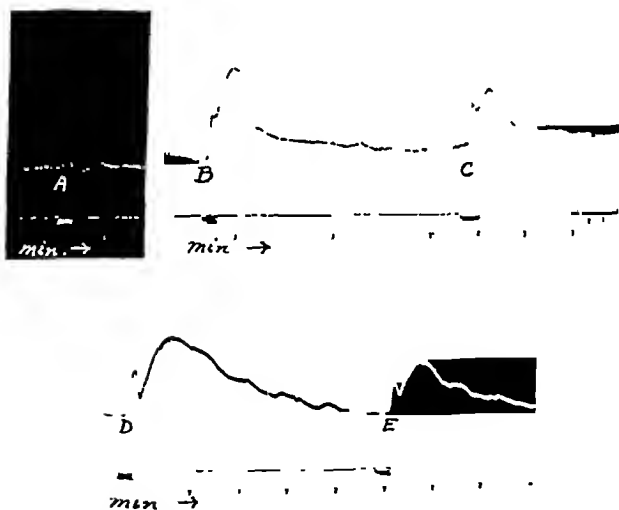


Fig. 6. Sensitization to the pressor action of adrenaline. 2 kg. cat. Sodium barbitone (1 g.). Doses of 1, 9 and 5  $\mu$ g. of adrenaline were given at A, B and C respectively. Following intravenous injection of S-*tert*-amyl *iso*-thiourea (0.1 g.), comparable rises of blood pressure could now be obtained with smaller doses of adrenaline as at D (1  $\mu$ g.) and E (0.5  $\mu$ g.).

acetone solution was used in the case of S-*tert*-amyl *iso*-thiourea salicylate), it was found that the initial pressor response could now be obtained with a smaller dose of adrenaline than previously (Fig. 6). If, for example, the same pressor effect could now be produced with 1/5th of the original dose of adrenaline, the degree of sensitization is described in Table 1 as 5. It was noticed in

some experiments where the height of the pressor response was unaffected that there was an increase in its duration. Often the pressor action was both increased and prolonged.

Experiments on the sensitization of dogs and cats to the pressor action of sympathomimetic amines other than adrenaline were also carried out. In four animals out of four, sensitization to the pressor action of *l*- $\alpha$ -hydroxy  $\beta$ -methyl amino-3-hydroxy ethylbenzene hydrochloride (neo-synephrin) was produced by S-methyl *iso*-thiourea, although the effect was more evident when S-ethyl *iso*-thiourea was given in addition. Sensitization to neo-synephrin by S-ethyl

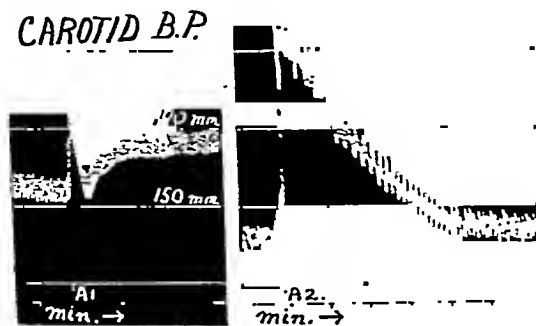


Fig. 7. Sensitization to the pressor action of neosynephrin. 11 kg. dog. Sodium barbitone (2.3 g.). Vagi cut. Doses of neosynephrin (0.5 mg. at  $A_1$  and  $A_2$ ) were given before and after administration of S-ethyl *iso*-thiourea (3 doses each of 0.1 g.).

*iso*-thiourea alone is illustrated in Fig. 7. With ephedrine, the results were less conclusive. Usually, following administration of the *iso*-thiourea, the rise of blood pressure after a dose of neosynephrin or ephedrine was increased but the duration of the rise was often shortened.

Since S-ethyl *iso*-thiourea regularly sensitized the whole animal to the pressor response of adrenaline, this particular derivative was used in experiments on isolated organs. In eight out of a series of eleven experiments on the perfused hindquarters of the rat, the injection into the perfusion cannula of S-ethyl *iso*-thiourea (10–100  $\mu$ g.) definitely sensitized the preparation to the vasoconstrictor action of adrenaline administered in the same way (Fig. 4). In the remaining three experiments the decrease in the rate of perfusion following a constant dose of adrenaline (usually 0.1  $\mu$ g.) was greater after the administration of the *iso*-thiourea than before, but the difference was not beyond the range of experimental error.

When treated with S-ethyl *iso*-thiourea, eight out of nine arterial strips taken from six animals and immersed in Ringer-Locke solution contracted more strongly in response to adrenaline (concentration 1 in  $10^6$  to  $10^7$ ) than previously (Fig. 5). Here also sensitization to the vasoconstrictor action of

adrenaline apparently takes place. Often the concentration of S-ethyl *iso*-thiourea employed (1 in  $10^3$  to  $10^4$ ) was not sufficient for it to have any direct action on the excised arterial spiral, although much weaker concentrations produce contraction of the smaller blood vessels in the whole animal.

#### *Effect on the heart and respiration*

S-methyl *iso*-thiourea depresses and slows the isolated perfused heart of the rabbit and cat, but cardiac depression is not obvious in the whole animal. The respiration is increased, apparently by 'stimulation of the medullary centre [McGeorge *et al.* 1942]. In most cases, the intravenous injection of the other *iso*-thiourea derivatives produced slowing of the heart beat and increased respiratory movements. With S-ethyl *iso*-thiourea, for example, doses of 12, 30, 15, 70 and 45 mg./kg. respectively slowed the heart by 54, 20, 10, 44, and 16 beats per min. Often, an initial increase in the frequency and amplitude of the respiratory movements was followed by a greater decrease in both.

#### *Effect on the isolated gut*

S-methyl *iso*-thiourea in a concentration of 1:600,000 produces a definite increase in the tone and strength of contraction of rabbit's intestine suspended in Ringer-Locke solution, this effect being somewhat diminished but not abolished by previous atropinization [McGeorge *et al.* 1942]. The effect of other *iso*-thioureas on isolated strips of rabbit and rat intestine was therefore tested. Similar results were obtained with S-ethyl and S-*iso*-propyl *iso*-thioureas (1:100,000) even when the strength of the atropine (up to 1:1000) was such that doses of acetylcholine had very little effect. The behaviour of S-*n*-butyl and S-*tert*-amyl *iso*-thiourea was more irregular. With the former compound an initial contraction was sometimes followed by relaxation of the intestine. In several of these experiments, adrenaline was given before and after the administration of the *iso*-thiourea. Although constant results were not obtained, there was no indication that the gut could be sensitized to the relaxing effect of adrenaline by treatment with these substances.

#### DISCUSSION

Perhaps the most interesting property of the *iso*-thiourea derivatives is their pressor activity. The first dose (0.005 g./kg.) of S-methyl, S-ethyl, S-*iso*-propyl and, occasionally, S-*tert*-amyl *iso*-thiourea may evoke a rise of blood pressure lasting 15-60 min. The pressor effect of the S-*n*-butyl derivative is, on the other hand, less regular and of short duration, and the contracting effects on the isolated intestine of both the S-*n*-butyl and the S-*tert*-amyl derivatives are less constant than those of the other three. The evidence at our disposal points to this pressor action having a peripheral origin in a direct stimulant effect of the drugs upon the muscle of the vessel walls, since both S-methyl and S-ethyl *iso*-thiourea cause vasoconstriction in the perfused pithed hind



limbs of the rat, and contraction of the isolated arterial spiral is induced by *S*-ethyl *iso*-thiourea. The contracting effect of these substances on the isolated intestine is further evidence of their acting directly on smooth-muscle and not, in the case of the blood vessels, through the mediation of the sympathetic nervous system.

It is conceivable that the sensitizing effect of these substances on the pressor action of adrenaline, which we have described, may contribute to their vasoconstrictor action when they are administered to the whole animal. Their pressor action cannot, however, be due entirely to this sensitization to adrenaline, since their contracting effect on arteries can be demonstrated, not only in the perfused hindlimb, but also in the isolated arterial spiral where the presence of circulating adrenaline is excluded. It is, of course, possible that the pressor action of the thiourea derivatives is due to some interference with the inactivation of adrenaline contained within the substance of the muscular wall of the blood vessel, but this hypothesis is not supported by our finding that the pressor activity of the *iso*-thiourea derivatives is in no way proportional to their sensitizing effect on the action of adrenaline. Whatever may be the cause of the sensitization to adrenaline, it is clearly of peripheral origin; it occurs in isolated tissues and in animals in which the vagi have been sectioned, and cannot, therefore, be attributed merely to a reduction in the normal vagal response to elevation of arterial blood pressure.

In a paper on the preparation of alkyl sulphonyl chlorides from *iso*-thioureas, Sprague & Johnson [1937] have stated: 'Several of these *iso*-thiourea combinations promise to prove of future physiological interest and their pharmacological study will receive immediate attention.' The only other references to the pharmacology of *iso*-thiourea derivatives found in the literature available to us have dealt with their possible effect on metabolism (hypoglycaemic action, catalysis of tissue oxidations).

#### SUMMARY

1. Observations have been made on the circulatory effects of *S*-methyl, *S*-ethyl, *S*-*iso*-propyl, *S*-*n*-butyl and *S*-*tert*-amyl *iso*-thioureas.
2. These substances raise the blood pressure, slow the heart and stimulate the respiratory movements. The pressor action is most pronounced in the case of the *S*-methyl, *S*-ethyl and *S*-*iso*-propyl derivatives. Contraction of blood vessels has been demonstrated, in the case of the *S*-methyl and *S*-ethyl derivatives, on the isolated pithed hindquarters of the rat perfused with Ringer-Locke solution. Hence it is thought that their effect on the blood pressure is due, at least in part, to a direct action on the blood vessels.
3. Repeated injections of any of these substances lead to a condition of tachyphylaxis, and at the same time the pressor action of other homologues is diminished or even abolished.

4. They enhance the pressor action of adrenaline in the whole animal.
5. Sensitization to the vasoconstrictor action of adrenaline by S-ethyl *iso*-thiourea has been shown to take place in the perfused pithed hindquarters of the rat and also in excised arterial spirals in Ringer-Locke solution, so that the blood vessel is the site of the process responsible for sensitization to the vasoconstrictor action of adrenaline. However, the *iso*-thioureas have a vasoconstrictor action which does not depend on this property.
6. S-methyl, S-ethyl and S-*iso*-propyl *iso*-thiourea cause contraction of isolated strips of atropinized gut, probably by a direct action on smooth muscle, but with the S-*tert*-amyl and, especially, the S-*n*-butyl derivatives, contraction of intestine is not regularly observed.

The expenses of this research have been defrayed in part by a grant from the Medical Research Council of New Zealand. We are also indebted to Mr C. L. Carter, M.Sc., A.I.C., for a micro-analysis, and to Mr G. H. Green, B.A., B.Sc., for some of the *iso*-thiourea derivatives used in the preliminary trials.

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## DISCHARGES FROM VESTIBULAR RECEPTORS IN THE CAT

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(Received 20 August 1942)

The electrical activity of the vestibular nerve has been studied by Ross [1936], who recorded impulses in single fibres in the frog and was able to distinguish the discharges due to the various parts of the labyrinth. Sand [1938] and Löwenstein & Sand [1936, 1940] have made similar records from the dog-fish and ray, but hitherto the Mammalia vestibular organs have not been investigated in this way. One reason for this is no doubt the great prominence of the cochlea activity in the 8th nerve of mammals and another the difficulty of reaching the vestibular fibres without interfering with the blood supply to the organ. In the present work this difficulty has been avoided by the use of a fine wire electrode thrust into the brain stem in the region of the vestibular nucleus so as to pick up impulses from the entering bundles of vestibular fibres. A disadvantage of the method is that the exact nature of the units which give rise to the electric charges must remain uncertain; but the records show the same general type of discharge as those found in the frog and seem to give a reasonable picture of most varieties of vestibular activity.

### METHOD

All the experiments were made on cats anaesthetized with dial, nembutal or chloralose or else decerebrated under ether. In the decerebrate preparation the cerebellum was removed as well as the cerebrum; in the others partial removal of the occipital bone gave access to the cerebellum and most of it was removed to expose the floor of the 4th ventricle. A spiral of silver wire coated with silver chloride was sewn to the scalp to form an indifferent electrode and the other was a fine enamelled silver wire (no. 44 S.W.G.) fixed to a rod which could slide in a ball and socket mounting fastened rigidly to the skull. With this arrangement (described in detail by Adrian & Moruzzi, 1939) the wire could be thrust into any part of the brain stem and would not be disturbed by movements of the head.

In some of the earlier experiments and sometimes as a control the vestibular organs were stimulated by turning the head by hand, leaving the body all the time in the prone position. As soon as it was clear that vestibular impulses

could be recorded in this way a swinging platform was arranged so that the whole animal could be turned laterally or tilted, without altering the position of the head in relation to the body (Fig. 1). The platform was hung at three points by parallel cords attached to spiral springs, the upper ends of which were fixed to the roof of the screening cage. The weight of the cat with the animal board, etc., was enough to extend the springs considerably, and they were flexible enough to allow the platform to be rotated horizontally through  $45^\circ$ , tilted laterally or in the long axis of the body or moved as a whole in the vertical or horizontal plane. This arrangement was fairly satisfactory though its range was limited and movements of constant velocity could not be studied. The nature of the suspension made it difficult to be sure of confining the movement of the platform strictly to one plane, but the amount of movement in a direction other than that intended was seldom enough to affect the record. The rate of movement was controlled by hand or by altering the moment of inertia of the system and allowing it to oscillate freely. A rough indication of the rate and extent of movement was given by a signal lever moved by a thread attached to one corner of the platform. In later experiments the reactions of the horizontal canals were analysed in more detail by mounting the animal on a turn-table which could be rotated at a constant speed, but the swinging platform was still used as the most convenient arrangement for the initial step of locating the vestibular discharges in the brain stem. With either arrangement the cat lay prone on the animal board with a mouth bar for the head and the body immobilized by sandbags.

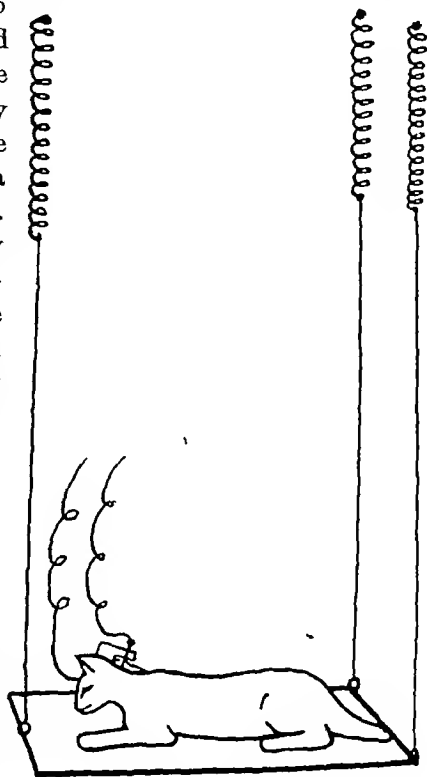


Fig. 1. Swinging platform for vestibular stimulation.

mounting the animal on a turn-table which could be rotated at a constant speed, but the swinging platform was still used as the most convenient arrangement for the initial step of locating the vestibular discharges in the brain stem. With either arrangement the cat lay prone on the animal board with a mouth bar for the head and the body immobilized by sandbags.

The usual procedure was to set up the animal on the platform and then to manipulate the electrode holder so that the wire was thrust about 1 mm. below the surface of the 4th ventricle in the region of the vestibular nucleus. As the wire penetrated the grey matter the platform was tilted and rotated to stimulate the vestibular organs. By listening to the potential changes reproduced by a loud speaker it was usually possible to detect vestibular activity

at many points near the entry of the 8th nerve. The area giving vestibular discharges was about 4 mm. long and 3 mm. wide in the position shown in Fig. 2. With the tip of the electrode just below the surface the sound was usually that of many units and was often evoked by more than one type of movement, but with the aid of the loud speaker it was nearly always possible

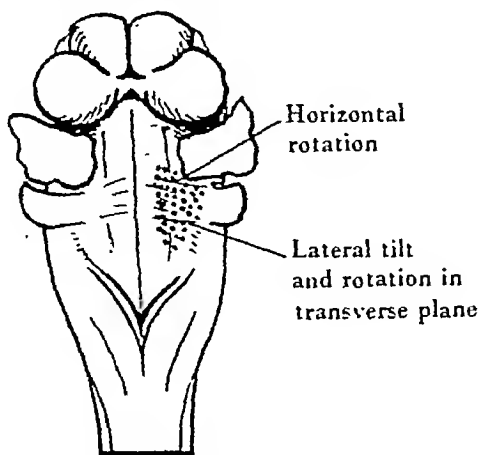


Fig. 2. Cat's brain stem after removal of cerebellum to show the region from which vestibular discharges can be picked up.

to adjust the electrode first to make one series of impulses appreciably louder than the rest and finally to make them appear alone without competitors in the background. Such a series would be found to depend predominantly on one kind of platform movement, and the relation of stimulus to discharge could then be studied in detail.

## RESULTS

### *Various types of discharge*

In Ross's analysis of the nerve discharges from the frog's labyrinth it was found that in some of the nerve fibres the discharge depended only on gross movement or on the position of the head in space, whilst in others it was produced by mechanical vibration. In the cat no vibration receptors have been detected. An electrode in the cochlea part of the nerve will of course pick up electrical activity due to sound vibrations, but no reactions either to sound or to mechanical vibration have ever been obtained from the region of the vestibular nucleus. Possibly a more thorough search would have revealed them, but it is more likely that in the cat the vestibular organs are not specially sensitive to vibration, the development of the cochlea having reduced the need for any other vibration receptors in the ear.

The responses to movement and position fall into two groups as in the frog. There is (a) the gravity-controlled type in which the discharge depends on the

could be recorded in this way a swinging platform was arranged so that the whole animal could be turned laterally or tilted, without altering the position of the head in relation to the body (Fig. 1). The platform was hung at three points by parallel cords attached to spiral springs, the upper ends of which were fixed to the roof of the screening cage. The weight of the cat with the animal board, etc., was enough to extend the springs considerably, and they were flexible enough to allow the platform to be rotated horizontally through  $45^\circ$ , tilted laterally or in the long axis of the body or moved as a whole in the vertical or horizontal plane. This arrangement was fairly satisfactory though its range was limited and movements of constant velocity could not be studied. The nature of the suspension made it difficult to be sure of confining the movement of the platform strictly to one plane, but the amount of movement in a direction other than that intended was seldom enough to affect the record. The rate of movement was controlled by hand or by altering the moment of inertia of the system and allowing it to oscillate freely. A rough indication of the rate and extent of movement was given by a signal lever moved by a thread attached to one corner of the platform. In later experiments the reactions of the horizontal canals were analysed in more detail by mounting the animal on a turn-table which could be rotated at a constant speed, but the swinging platform was still used as the most convenient arrangement for the initial step of locating the vestibular discharges in the brain stem. With either arrangement the cat lay prone on the animal board with a mouth bar for the head and the body immobilized by sandbags.

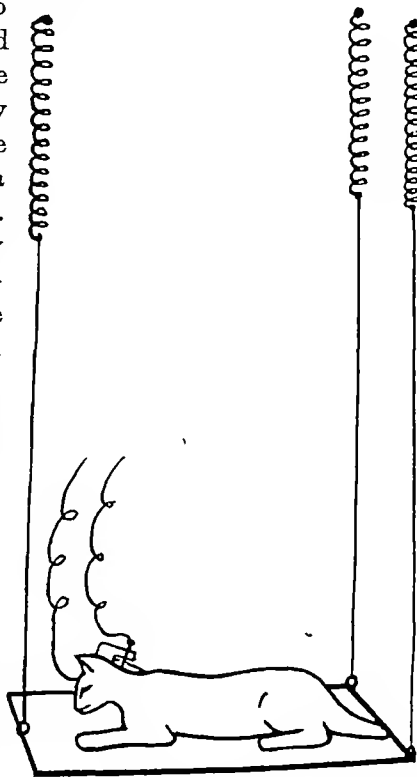


Fig. 1. Swinging platform for vestibular stimulation.

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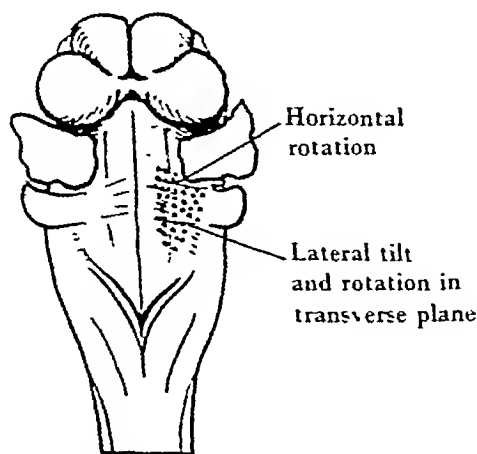


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The responses to movement and position fall into two groups as in the frog. There is (a) the gravity-controlled type in which the discharge depends on the

position of the head in space and is evoked by tilting into the stimulating position as well as by linear accelerations, and (b) the rotation-controlled type, where the response is not affected by the position of the head and is determined entirely by angular acceleration. As regards group (a) it has always been easy to find gravity-controlled discharges produced by tilting the head laterally, so that the side under examination is the lower; discharges controlled by the fore-and-aft position of the head have been less common but have been found in four out of six animals. As regards group (b), discharges in response to rotation in the horizontal and transverse planes have been easy to find. Those in response to rotation in the median plane have been more difficult, but like the response to fore-and-aft tilting they have usually been found in the end. The gravity-controlled type of discharge will be dealt with first, since the relation between stimulus and response is more straightforward.

### *Gravity-controlled discharges*

*Lateral tilt.* Typical records of a discharge controlled by the lateral tilt of the head are shown in Fig. 3. The tip of the electrode was 1.5 mm. below the surface of the brain stem in the middle of the striae acousticae on the right side. In this position it picked up a series of large potential spikes with a frequency of about 6 per sec. when the head was level. When the head was tilted to the left (i.e. with the left cheek down) the discharge ceased, and when it was tilted to the right the frequency increased, reaching 95 per sec. for a tilt of  $20^\circ$  from the level. The position of the head in each record is given by the signal line: the curves in Fig. 4 show the relation between the tilt and the frequency after the first second in this and in other preparations.

The records in Fig. 3 are from a decerebrate animal. In several cats under dial and nembutal discharges of this type have not reached higher frequencies than 40–50 per sec., and in some units the discharge has not started until the head has been tilted a few degrees down towards the side under examination, though it is more usual to find a discharge at a low frequency when the head is level (as in Fig. 3). In no case has there been an increase in frequency when the tilt has been in the opposite-sense, i.e. raising the side under examination and lowering the other, and tilting in the median plane has rarely produced more than a slight effect on these units unless the snout has been turned up through a considerable angle.

Fig. 5 shows what happens when the head is tilted sideways through  $15^\circ$  and held in that position for 33 sec. The frequency declines slowly, for the receptor is of the slowly adapting type and the discharge depends principally on the position of the head and is not much affected by the time it has been in that position. It is also very little affected by the rotational movement as such, for rotation through a limited angle does not excite unless it brings the head into a position adequate to produce a steady discharge. Evidently we are



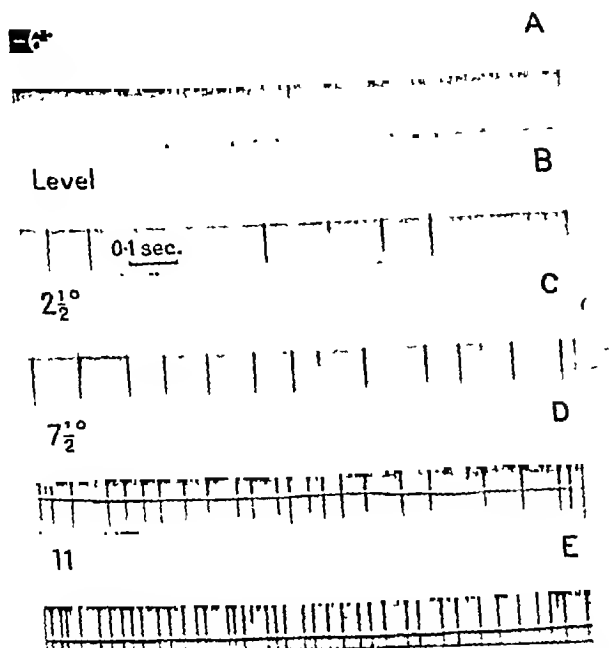


Fig. 3. Records of a gravity-controlled discharge from the right labyrinth signalling the lateral tilt of the head to the right. Decerebrate cat. The inclination of the transverse axis of the head is shown by the white signal line in each record. Time marker (black line in B) gives 0.1 sec.

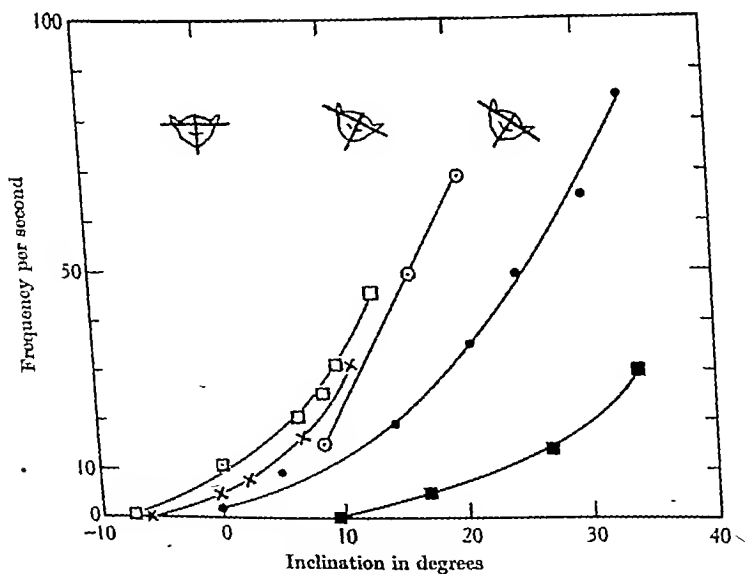


Fig. 4. Relation between lateral tilt and frequency of discharge in units from several animals: insets show position of head when tilted to the left.

dealing with a receptor which is stimulated when the gravitational pull acts on it in certain directions. The most likely receptor is the saccular otolith organ, and if so, it is stimulated when the otolith begins to pull away from the

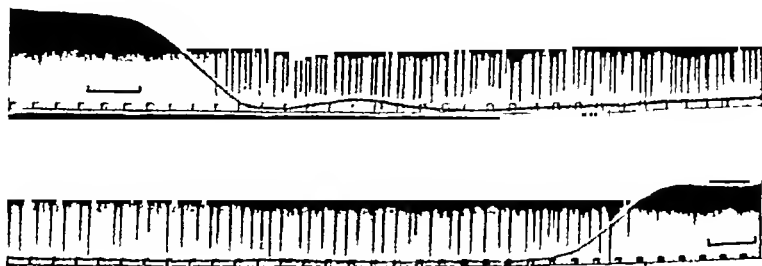


Fig. 5. Beginning and end of a discharge produced by a lateral tilt of  $15^\circ$  (same unit as in Fig. 3). The position was maintained for 33 sec. Initial frequency 68 per sec., final 40 per sec. Time marker (black line) gives 0.1 sec. in these and all other records.

macular endings, as Magnus and his colleagues concluded from other lines of evidence [Magnus, 1924].

Since these discharges are due to the gravitational pull on a receptor mechanism they ought to be produced by linear acceleration in certain directions as well as by particular positions of the head. In every case it was

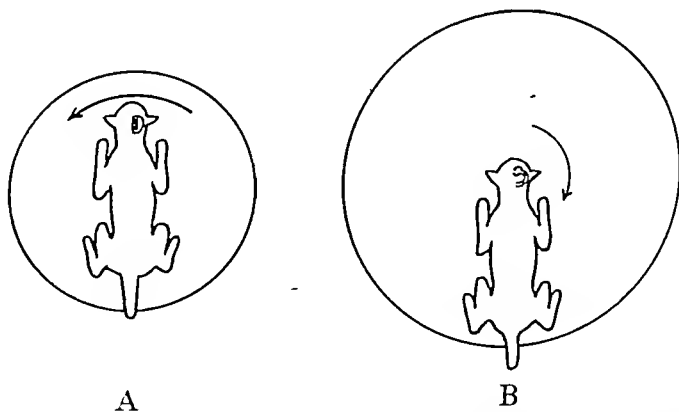


Fig. 6. Illustrating (A) stimulation of right lateral gravity receptor by acceleration of head to left when the axis of rotation is in the centre of the trunk. When the axis passes through the head (B) the gravity receptors are not stimulated. The horizontal canal is stimulated by rotation to the right.

found that movement sideways in the horizontal plane could be made to evoke or increase the discharge. The stimulating effect occurs only when there is an acceleration away from the side examined, so that if the electrode is in the

right vestibular nucleus a discharge will occur when the head is accelerated to the left and vice versa. This can be seen by allowing the platform to swing from side to side without tilting or rotating, but it is shown very clearly when the animal is on the turn-table and is rotated in the horizontal plane: rotation about an axis passing through the head (Fig. 6 B) has no effect on a gravity-controlled discharge, but if the axis passes through the trunk a discharge occurs whenever the head is accelerated to the left (Fig. 6 A). Evidently an acceleration to the left would have the same effect as a gravitational pull to the right whatever the precise mechanism of the gravity receptor may be.

Two of the discharges controlled by lateral tilt and lateral acceleration have responded also to rapid fore-and-aft and vertical up-and-down acceleration. Both were modified by the fore-and-aft tilt of the head though not so much as by the lateral tilt. As both were single unit discharges we must suppose that the saccular endings (if these are responsible) can sometimes be stimulated by a pull of the otolith in the same plane as the macula, though a pull away from the macula is always more effective.

*Controls.* Before we can be sure that these discharges really represent the activity of a vestibular organ we must rule out the possibility that they are due to a movement of the electrode in the brain stem. When the wire is thrust into the floor of the 4th ventricle it must cause some injury, and the penetration often evokes trains of potential spikes which last for a few seconds and are presumably caused by damage to nerve cells or fibres. Such injury discharges, like those in the cerebrum, can often be modified by slight movement of the nervous tissue relative to the electrode, and though the modification is usually no more than a change in the size of the recorded potentials there is sometimes a rise and fall of frequency as well. Thus a sideways tilt might cause an increased frequency of discharge, not because the electrode was recording the signals of a gravity receptor, but because it was altering the deformation of injured nervous tissue.

There are two reasons for rejecting such a view, first that the responses usually persist unaltered for an hour or more, and second that they are only evoked by the one kind of movement, a gravity-type discharge from the right vestibular region being increased only by tilting down to the right or by linear acceleration to the left. No such permanence or specificity would be likely to occur in an injury discharge, and it can scarcely be a coincidence that the movements which stimulate are precisely those assigned by Magnus and de Buriel to the lateral gravity receptors. On several occasions, also, a discharge evoked by tilting the whole platform has been evoked just as clearly by tilting the head alone, though the shift of the brain stem would probably differ considerably. It does seem possible, however, that injury may be responsible for one feature of the records, namely, the occasional appearance of brief high-frequency groups of impulses instead of single spikes. These have not been examined in detail, but they suggest some interference with the normal mechanism of the nerve fibres.

Controls have also been made from time to time to ensure that the discharges ascribed to the vestibular apparatus are not due to receptors belonging to the 5th nerve stimulated by air currents on the ears or vibrissae when the head is turned. Discharges from the face, jaws and vibrissae are easily picked up in the brain stem, but the electrode must be deeper and further from the midline, and the response is clearly related to stimulation of the receptors of the face and not to those of the vestibular organs.

*Fore-and-aft tilt.* Persistent discharges controlled by turning the snout of the animal upwards have been found in four animals but only after considerable search. This may be due merely to the arrangement of the platform which made

it difficult to produce an upward tilt in which the line of the jaw made an angle of more than  $10^\circ$  with the horizontal; such a tilt may well have been inadequate to stimulate the majority of the receptors. The four examples were probably from gravity and not rotation receptors, for angular acceleration did not stimulate unless it brought the head into the upturned position; linear acceleration sideways or forwards was moderately effective in one animal, but linear acceleration downwards was effective in all. This would agree with Magnus's view that the utricular otolith organ is stimulated by a movement or position which pulls the otolith away from the macula on which it rests.

In several preparations discharges have been found in response to vertical upward or downward acceleration and to nothing else. These may well have come from gravity receptors which would have responded to a tilt of sufficient extent but were not stimulated by the positions available with the platform. It is doubtful whether the utricular otolith organ should be held responsible for the discharges due to upward acceleration as well as for those to downward; the former may possibly come from the dorsal lobe of the saccular organ, since it faces downwards instead of upwards and its nerve fibres run with those from the utricle [Oost, 1918; de Burlet & de Haas, 1923].

#### *Rotation-controlled discharges*

These are the discharges which are produced by angular acceleration of the head in various planes and are not affected by its initial or final position with respect to the earth. They are presumably derived from the semicircular canals,

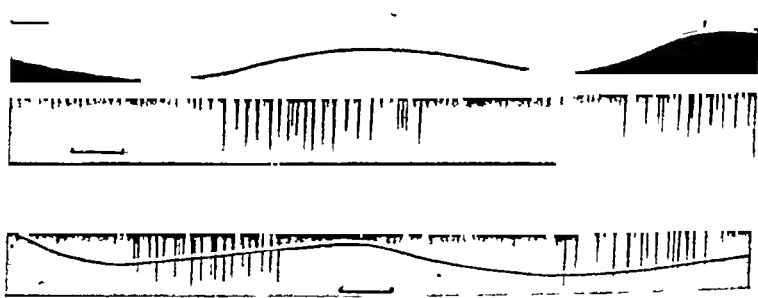


Fig. 7. Effect of lateral tilting on a rotation-controlled discharge (unit responding to rotation in transverse plane). Decerebrate cat. The initial position of the head does not affect the response.

for it is found that for each single-unit discharge the most effective stimulus is a rotation in a particular plane, rotation in planes at right angles having little or no effect. The difference between the gravity type and the rotation type can be seen by comparing Fig. 3 and Fig. 7, which shows a unit responding to rotation in the transverse plane (lateral tilting). As in Fig. 3 the vertical position of the signal line shows the tilt of the head, and it will be seen that in this case the amount of tilt is immaterial and the movement all important.

*Rotation in different planes.* With the simple tilting platform arrangement it was difficult to test the effect of rotations in very many planes, but there is no doubt that for some units horizontal rotation is a far more effective stimulus than rotation in any other plane. For other units, rotations in the transverse or in the median plane of the body were the most effective, but with these two planes the best position was less sharply defined. In every case the cat lay prone with the head in the normal position, i.e. with its long axis inclined about  $45^\circ$  from the horizontal. Minor variations in head posture made little difference, but a sideways tilt would sometimes complicate the result.

In all the units which have been controlled by horizontal rotation the discharge has been stimulated by rotating the head towards the side from which the unit comes and suppressed by rotating it towards the opposite side (Fig. 8 A). Thus for the horizontal canal the stimulating direction is with the ampulla trailing. In the units controlled by rotation in the transverse plane the discharge is increased by rotating the top of the head towards the side from

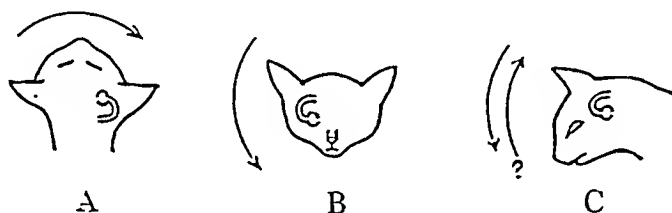


Fig. 8. Direction of rotation which stimulates the three canals. See next.

which the unit comes, so that if we are dealing with the posterior canal this is stimulated by ampulla leading rotation (Fig. 8 B). These results support the accepted view of the canal mechanism. With rotation in the median plane (anterior canal) the results are less clear. In the frog, Ross found that the anterior canal was stimulated by ampulla leading (snout down) rotation and Owenstein & Sand found the same with the ray, but in the present experiments there have been several clear examples of units apparently of the rotation-controlled type stimulated by tilting the snout up as well as units responding to tilting it down (Fig. 8 C). The imperfections of the apparatus have made it difficult to decide whether these were really of the rotation- or the gravity-controlled type. It has already been noted that the gravity receptors for lateral tilt will also react to horizontal rotation if it produces some lateral translation of the head. In the same way therefore a rotation in the median plane involving an upward or downward translation of the head might stimulate the utricular otolith organs and these may have caused the discharges mentioned above. In any case it is presumably useful for the vestibular apparatus to signal rotation in either direction.

The discharges controlled by horizontal rotation and fore-and-aft tilt have usually been found further forward in the brain stem than those controlled by lateral tilt (gravity or rotation type), the former being near the oral and the latter near the aboral margin of the striae acusticae (cf. Fig. 2). As regards the specificity of the stimulus: the discharge to horizontal rotation is usually quite unaffected by rotation in the transverse or median plane, but rotation in one of these two will often produce some effect on a discharge which is mainly controlled by rotation in the other. Löwenstein & Sand found that the anterior and posterior canals in the ray would respond to rotation about any of the three primary axes, but in the cat horizontal rotation has had very little effect on the units stimulated by transverse or median plane rotation.

Magnus suggested that linear acceleration forwards might be expected to stimulate the horizontal canal. In the present experiments it has never produced more than an occasional impulse in a unit reacting to horizontal rotation, and linear acceleration sideways has been equally ineffective.

*The resting discharge.* Rotation-controlled discharges have been described for the frog by Ross and for the dogfish and ray by Löwenstein & Sand, and there is one point on which their findings are not in complete agreement. Ross says that in the frog a small proportion of his single-fibre preparations showed a persistent resting discharge which 'could be momentarily suspended by a movement in a direction opposite to that which stimulates the end organ'. But Löwenstein & Sand find such a discharge in all their preparations of nerve fibres from the horizontal canal of the dogfish. Since it is suppressed by rotation in the non-stimulating sense they regard it as an important feature of the receptor mechanism, for as they point out it provides a means by which a single receptor can signal rotation in either direction instead of in one direction only.

In the cat's brain stem under the conditions of these experiments the rotation-controlled discharges are very often of the persistent type, continuing at a low frequency when the head is at rest and ceasing only with rotation in the non-stimulating sense; but in some units there has been no resting effect, although rotation through a very small angle has been enough to evoke a discharge. Examples of the two kinds of response are shown in Fig. 9. In Fig. 9 A the discharge is from the right posterior canal; it continues at 9 per sec. during rest, is suppressed by tilting the head to the left, and is increased by tilting it to the right. In Fig. 9 B the discharge (also from the posterior canal) is absent at rest and appears only with rotation in the one sense. In Fig. 9 C the discharge varies in frequency, but is never completely suppressed.

What proportion of the receptors give resting discharges is hard to say. About two-thirds of the present series have done so, though it is naturally easier to locate a unit which is always in action than one which is quiet until the head is turned. There is evidently a wide variation in the excitability of

the different units: the frequency of the resting discharge has varied from about 2 to 30 per sec., and there is probably a continuous gradation from the receptors which need a movement to make them discharge at all (cf. Fig. 9 B) to those which continue at a fairly high frequency all the time (cf. Fig. 9 C). The nature and depth of the anaesthetic has no obvious effect on the prevalence of resting discharges, decerebrate cats with a high blood pressure showing no



Fig. 9. Units with and without a resting discharge. A and B are units controlled by rotation in transverse plane (posterior canal). In A there is a resting discharge at 9 per sec. suppressed by tilt to the left and increased by tilt to the right. In B there is only a discharge on tilting to the right. Both decerebrate cats. C is a unit controlled by rotation in median plane with a discharge which is never completely suppressed. Cat under nembutal.

greater proportion than cats under deep dial; and a mild degree of hyperventilation, an injection of adrenalin or of intravenous saline has produced no definite modifications of the response.

*The relation of stimulus to response: after-discharges, etc.*

With the swinging platform a rotation through a small angle involves an acceleration followed immediately by a deceleration, and this is usually followed by a similar movement back again to the initial position. Though this rapid sequence of changes complicates the picture it is not unlike the sequence to which the canals are subjected in life, for the turning of the head must always involve an acceleration and deceleration with little or no interval between. The records in Figs. 10, 11 and 13 show what kind of discharge comes from a canal in these circumstances. The frequency increases during the acceleration; during the deceleration and the movement back it falls to zero or to a value lower than that of the resting discharge. The maximum frequency is evidently related to the acceleration, but owing to the deceleration which follows it is impossible to say how rapidly the receptors would become adapted to the stimulus or indeed to decide what it is that stimulates them.

To investigate this a turn-table was substituted for the swinging platform. It could be rotated at a fairly uniform rate which was recorded by an interrupted light signal. The electrical changes from the brain stem were led off by connecting the electrodes to a length of light flex hung from the ceiling over the centre of the turn-table. This allowed the platform to make up to 20 revolutions without undue twisting or untwisting of the wires. Only the horizontal canal discharges were investigated in this way, since it was difficult to arrange the cat in anything but the prone position.

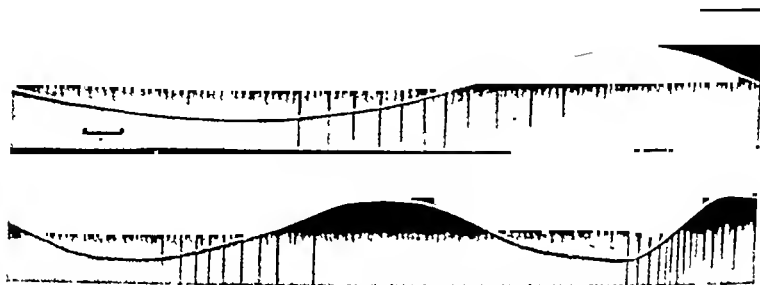


Fig. 10. Discharges from the posterior canal (rotation in transverse plane) showing effect of different rates of swing. Decerebrate cat (same as Fig. 9 B).



Fig. 11. Discharges from horizontal canal. A, unit from left side with resting discharge. Signal shows swing to the right and back. Cat under nembutal. B and C, unit from left side with resting discharge, showing effect of horizontal swings at different rates. Cat under dial.

The results obtained with the turn-table agree with Ross's for the frog and support his view of the receptor mechanism, which is that the receptors adapt slowly, the excitation varying with the deflexion of the cupola. It is found, as in the frog, that the frequency rises to a maximum as the turn-table accelerates and then declines slowly as the rotation is continued at constant speed. With a speed of rotation of 1 rev. in 2 sec. attained in the first  $\frac{1}{2}$  sec. the discharge does



not revert to its resting level for 10–15 sec. With slower acceleration and rotation the maximum frequency is lower and the discharge briefer: but with any turning movement in which the acceleration is not immediately followed by

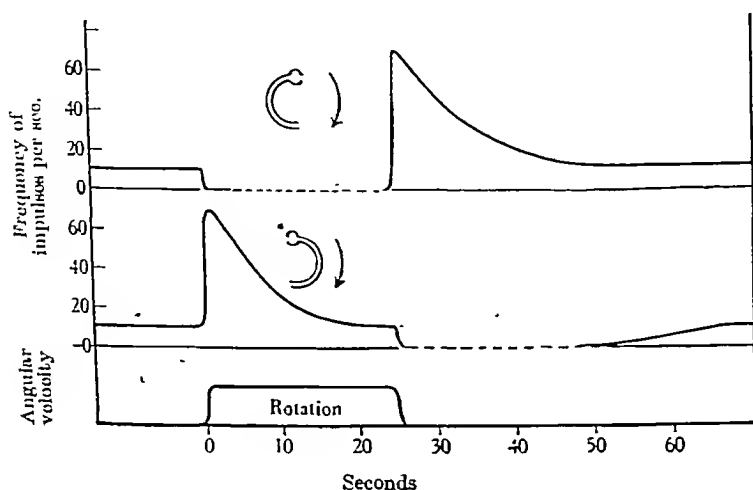


Fig. 12. Average time course of after-discharges and silent periods when acceleration and deceleration are separated by an interval of steady rotation. Both of the units are shown as having a resting discharge and as giving effects of maximum duration (such as would follow 25 sec. rotation at 0.5 rev./sec.). The vestibular discharge gives a misleading picture of the head movement (cf. Fig. 13).

TABLE 1

Preparation	Unit	Resting discharge per sec.	Longest after-discharge sec.	Longest suppression of resting discharge (until complete return) sec.
Cat A (dial)	1	25	20	25–35
	2	0	12	—
	3	1	9	30
	4	7	8	15
Cat B (dial)	1	0	10	—
Cat C (nembutal)	1	10	15–25	25–30
	2	6	20	20–30
	3	4–5	15–20	15–20
	4	0	15	—
Cat D (decerebrate)	5	9	8	12
	1	14	16	25–30
	2	3–6	12	15
	3	0	10–15	—

When two figures are given these are the values obtained in different trials.

a deceleration the increased activity outlasts the period of acceleration by several seconds.

If the rotation is stopped during the discharge there is an immediate fall in frequency to zero or to a value lower than that of any resting discharge which

was present initially. Again, the effect varies with the speed of deceleration, but with a sudden stop there is usually a complete suppression of all activity and the resting discharge does not return for 10-30 sec. Thus the angular acceleration causes an excitation which takes some time to die down, and the deceleration has the reverse effect, suppressing all activity for an even longer period.

Similar effects are shown by rotating the head in the reverse, or non-stimulating sense. If the units are from the right side so that angular acceleration to the right stimulates the receptors, then angular acceleration to the left suppresses any resting discharge there may be. When rotation to the left has settled down to a constant speed the discharge builds up again to the resting frequency within 10-30 sec. And if at any time the reversed rotation is stopped the discharge immediately rises to a high frequency which declines gradually for the next 10-15 sec. Thus in its action on the rotation receptors angular acceleration to the right has the same effect as angular deceleration to the left, and deceleration to the right the same as acceleration to the left.

The results are summarized in Fig. 12, and the data for several experiments are given in Table 1. The after-effects of rapid accelerations and decelerations persist for 10-30 sec. and there is not much variation from one unit to another. Briefer effects could be obtained by smaller changes in velocity, but it did not seem likely that the after-effects would last much more than 30 sec. whatever the intensity of the stimulus. It may be significant that a resting discharge was present in the two units which gave some of the longest after-effects, but those with no resting discharge or with a very low frequency do not differ much from the others on the list. The uniform duration of all these responses suggests that they are determined by some relatively stable property of the semicircular canal mechanism such as the rate of subsidence of endolymph currents or the rate of return of the deflected cupola. They will evidently account for many of the reactions of the intact animal to rotation and theories of the canal mechanism based on these reactions ought equally to account for the main features of the receptor discharge.

#### *The receptor mechanism of the canals*

It is generally held that the excitation of a canal depends on a deflexion of the cupola by the flow of endolymph past it. This is enough to explain the excitation which accompanies acceleration or deceleration, since both will certainly produce a flow of endolymph, but something more is needed to explain the after-effects. It might be (a) that the movement of fluid outlasts the period of acceleration and of deceleration, or (b) that owing to its structure, etc. the deflected cupola takes some time to return to the mid-position when the deflecting force (i.e. the endolymph current) ceases, or a combination of (a) and (b). It is unlikely that (a) is the sole cause, for the flow of endolymph can scarcely continue for 10 sec. or more after rotation is stopped. Thus (b) is the

most likely cause of the after-effects. In any case we may reasonably assume an immediate deflexion of the cupola during accelerations and decelerations and a slow return to the mid-position when these have ceased.

If we suppose in addition that the canal receptors are slowly adapting like those of the saccule and utricle the main features of the nervous discharge are easily understood. Angular acceleration followed immediately by deceleration would give no more than a brief deflexion of the cupola and a brief discharge, and angular acceleration followed by rotation at a constant speed would give a longer discharge declining slowly as the cupola returned to its undeflected position. If the rotation was stopped abruptly the endolymph would flow in the opposite direction, the cupola would be deflected by it in the non-stimulating sense and would again return slowly to the mid-position. The opposite deflexion and gradual return will explain the silent period and the gradual return of the resting discharge, for where a resting discharge is present the mid-position must be one of slight excitation.

This view of the receptor mechanism has been dealt with already by Ross. It implies that the suppression of the discharge by deceleration, etc., is not due to any special inhibitory process acting on the receptors but is merely due to the removal of the stimulus. The physical properties of the system favour such an idea, for angular deceleration would be likely to cut short the stimulating effect of angular acceleration and acceleration in the opposite sense would deflect the cupola away from the positions which stimulate. But although the movements of the cupola seem enough in themselves to explain the main features of the discharge, it may be that there are also minor effects due to changes in the receptors. Many other kinds of receptor show a silent period following activity—for instance, when a stretched muscle spindle is released the impulse frequency falls to zero and the resting discharge, if present initially, does not return for several seconds. Here the removal of the load will restore the initial conditions, but there is no reason to believe that it will go beyond this and produce a state in which the receptor is exposed to a smaller stimulus than that afforded by the initial conditions. In fact there are cases in which a silent period following increased activity seems to depend on a fall of excitability in the receptors as well as cases in which it depends on the diminution of the stimulus which causes the resting discharge. Sometimes both kinds of effect may be found within a group of similar receptors, e.g. those of the cat's vibrissae [Fitzgerald, 1940].

With the canals there is reason to think that the suppression of a resting discharge sometimes depends on a fall of excitability as well as on a failure of the stimulus. For instance, it has been pointed out that the periods of diminished activity after deceleration are on the whole longer than those of increased activity after acceleration. This suggests a fatigue effect, a fall of excitability following activity added to the effect produced by the deflexion of the cupola

was present initially. Again, the effect varies with the speed of deceleration, but with a sudden stop there is usually a complete suppression of all activity and the resting discharge does not return for 10–30 sec. Thus the angular acceleration causes an excitation which takes some time to die down, and the deceleration has the reverse effect, suppressing all activity for an even longer period.

Similar effects are shown by rotating the head in the reverse, or non-stimulating sense. If the units are from the right side so that angular acceleration to the right stimulates the receptors, then angular acceleration to the left suppresses any resting discharge there may be. When rotation to the left has settled down to a constant speed the discharge builds up again to the resting frequency within 10–30 sec. And if at any time the reversed rotation is stopped the discharge immediately rises to a high frequency which declines gradually for the next 10–15 sec. Thus in its action on the rotation receptors angular acceleration to the right has the same effect as angular deceleration to the left, and deceleration to the right the same as acceleration to the left.

The results are summarized in Fig. 12, and the data for several experiments are given in Table 1. The after-effects of rapid accelerations and decelerations persist for 10–30 sec. and there is not much variation from one unit to another. Briefer effects could be obtained by smaller changes in velocity, but it did not seem likely that the after-effects would last much more than 30 sec. whatever the intensity of the stimulus. It may be significant that a resting discharge was present in the two units which gave some of the longest after-effects, but those with no resting discharge or with a very low frequency do not differ much from the others on the list. The uniform duration of all these responses suggests that they are determined by some relatively stable property of the semicircular canal mechanism such as the rate of subsidence of endolymph currents or the rate of return of the deflected cupola. They will evidently account for many of the reactions of the intact animal to rotation and theories of the canal mechanism based on these reactions ought equally to account for the main features of the receptor discharge.

#### *The receptor mechanism of the canals*

It is generally held that the excitation of a canal depends on a deflexion of the cupola by the flow of endolymph past it. This is enough to explain the excitation which accompanies acceleration or deceleration, since both will certainly produce a flow of endolymph, but something more is needed to explain the after-effects. It might be (a) that the movement of fluid outlasts the period of acceleration and of deceleration, or (b) that owing to its structure, etc. the deflected cupola takes some time to return to the mid-position when the deflecting force (i.e. the endolymph current) ceases, or a combination of (a) and (b). It is unlikely that (a) is the sole cause, for the flow of endolymph can scarcely continue for 10 sec. or more after rotation is stopped. Thus (b) is the



away from the stimulating position. It has also been found that in the earliest stages of the silent period following abrupt deceleration a rapid to-and-fro movement will produce no discharge although the turn-table is rotated through  $45^\circ$  or more. A few seconds later rotation through a smaller angle will give a brief discharge and the threshold stimulus becomes less and less as the resting discharge builds up again. This gradual increase in sensitivity may be due principally to the gradual return of the cupola after its deflexion away from

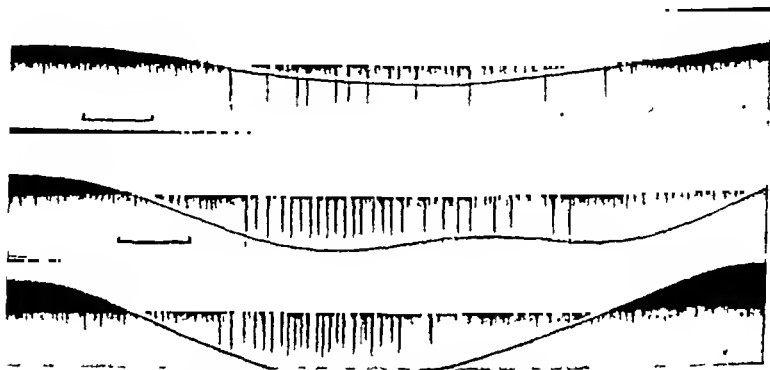


Fig. 13. Discharge from the anterior canal (rotation in median plane), showing effect of brief downward tilting of the head. The vestibular discharge gives an accurate picture of the head movement (cf. Fig. 12).

the mid-position; this would certainly account for the smaller and smaller angle of movement which will stimulate. But unless there is also a fall of excitability in the receptors themselves it is difficult to account for the complete failure to arouse a discharge in the early stages. But such evidence is scarcely conclusive and the point was not investigated in detail, as there is no reason to doubt that the deflexion of the cupola is the main factor in determining the production or suppression of a discharge from the receptors.

### DISCUSSION

The foregoing results have not shown any marked difference between the cat's vestibular apparatus and that of the frog or fish. There are the gravity receptors to signal the posture and linear acceleration of the head and the rotation receptors to signal turning movements, and all of them react in a manner which is consistent with the structure of the sense organs and with the reactions which it produces in the intact animal. Thus the duration of the after-effects when rotation is stopped is of the same order as that of the post-rotatory nystagmus in man; the planes of rotation which are most effective for the different units correspond more or less with those of the three canals, etc.



## SUMMARY

1. The impulses from vestibular receptors can be studied in the cat by a fine wire electrode thrust into the brain stem in the region of the vestibular nucleus. The results are in general agreement with those from cold-blooded vertebrates.

2. Discharges in single units belong to one of two main types, gravity-controlled and rotation-controlled. The former depend on the position of the head in space, the latter only on angular accelerations or decelerations. Discharges controlled by horizontal rotation and by the tilt of the head in the median plane are found near the oral border of the striae acusticae, those controlled by lateral tilt and by rotation in the transverse plane are nearer the aboral border. Responses to vibration have not been found.

3. The gravity receptors are increasingly stimulated as the head is tilted out of its normal position and become very slowly adapted to the stimulus. They react to linear accelerations as well as to the pull of gravity.

4. The rotation receptors are stimulated by angular acceleration in the horizontal, median or transverse plane or by deceleration of steady rotation in the opposite sense. For the horizontal canal the stimulating sense is with the ampulla trailing, for the posterior with the ampulla leading. With rotation in the median plane some units have reacted to snout up and some to snout down movement, but some of these may have been gravity receptors reacting to linear acceleration.

5. Quick turns of the head are signalled by brief discharges which coincide with the movement, but an acceleration not followed at once by deceleration gives a discharge lasting up to 25 sec. Equally long after-discharges follow deceleration from steady rotation in the non-stimulating sense.

6. The majority of the rotation receptors, though not all, give persistent low-frequency discharges when the head is at rest. These are suppressed by acceleration in the non-stimulating sense and by deceleration of steady rotation in the stimulating sense, and it may be as long as 30 sec. before the resting discharge is fully restored.

7. These results support Ross's view that the canal receptors adapt slowly, the stimulus being proportional to the deflexion of the cupola. With a brief turn the reversal of the endolymph flow brings the cupola back at once to the mid-position; with continued rotation the deflected cupola is brought back slowly by elastic forces and is then deflected the other way when rotation is stopped. The resultant suppression of activity may be reinforced by fatigue.



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## THE EFFECT OF BONE DYSPLASIA (OVERGROWTH) ON CRANIAL NERVES IN VITAMIN A-DEFICIENT ANIMALS

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This report is a continuation of one dealing with the wider problem of skeletal changes produced in young animals by vitamin A-deficient diets and their effect on the central nervous system [E. Mellanby, 1941]. An account is given here of overgrowth of bone in the region of the cranial nerves and its effect upon them, with the exception of the VIIIth nerve, which was dealt with in detail in a previous paper [E. Mellanby, 1938].

Degeneration of the optic nerve, the trigeminal and the auditory and vestibular divisions of the VIIIth nerve resulting from vitamin A and carotene deficiency in the diet were described in 1933 and 1934*a* [E. Mellanby]. It was thought at that time that these degenerative changes, together with others in the brain stem and spinal cord, were due mainly to the abnormal metabolism associated with vitamin A deficiency having a direct, destructive effect on certain nerve cells. A detailed study of the cochlea and vestibule made it clear, however, that the destruction of the VIIIth nerve was secondary to bone overgrowth. It was soon obvious that the degenerative changes in other cranial nerves and indeed of some peripheral nerves were associated with pressure resulting from abnormal bone growth [Mellanby, 1938, 1939*a, b*].

### METHODS

Litters of puppies from 6 to 10 weeks old were fed on basal diets of the following type: separated milk powder 20 g., cereal (usually white bread) 100-300 g., lean meat 15-20 g., yeast 3-12 g., peanut or olive oil 10 ml., sodium chloride 1-2 g., lemon or orange juice 5 ml., irradiated ergosterol (vitamin D<sub>2</sub>) 1000-2000 i.u.

There is a small amount of vitamin A in this diet, but not sufficient to prevent depletion of the body. Under the conditions of these experiments the calcium in the diet is not high enough for optimal bone formation when growth is rapid, but increasing the calcium modifies, though it does not prevent, the effects



any one point and subjects such nerve fibres to mechanical pressure. In the +A animal a number of bundles of nerve fibres traverse a single passage in the cribriform plate, but in the -A animal they seem to be broken up into individual bundles by the encroaching and irregular bone overgrowth; some of the bony passages in the plate are so narrow that even these smaller nerve bundles passing through them are pinched, as can be seen in Figs. 2*b* and 3*b* (see *N*). The compressed nerve can be seen in Fig. 1, which shows high-power photomicrographs of nerve sections in +A and -A animals respectively just before passing, during passage, and after passing through the cribriform plate towards the brain. The sections are stained to accentuate the appearance of the nuclei of the sheaths of these non-medullated nerve fibres. Fig. 1*b* (ii) shows that in the -A animal the nuclei are much more crowded during the passage of the nerves through the plate, indicating squeezing of the nerve at this place, whereas both before and after passage the nuclei are more dispersed and similar to the unsqueezed appearance of comparable sections in the +A animal (Fig. 1*a*). In spite of the pressure suffered, these particular nerves have not been completely destroyed, although it may be that other bundles of nerves or some nerves in any bundle have degenerated and disappeared. No histological preparations have been made which show whether changes are present in the axis cylinders of these non-medullated nerves.

In this, as in other situations, changes are sometimes observed in the dura mater and subarachnoid space in -A animals. The dura mater, which accompanies the nerve bundles in part of their passage through the cribriform plate, is thickened and may add to the pressure effect on the nerve bundles referred to above. The subarachnoid space of the +A animal contains much nerve tissue and some other connective tissue elements, whereas in the -A animal it seems relatively free from such tissues. The olfactory nerve fibres which pass across it to the olfactory lobe of the brain are broken up into much smaller bundles and appear to be greatly reduced in number, an indication that many fibres have been killed by the overgrown cribriform plate.

There is, however, one other factor to be considered in regard to the reduction in olfactory nerve fibres crossing the subarachnoid space towards the olfactory lobe of the brain. This is the raised intracranial pressure in some of these -A animals [E. Mellanby, 1939*a*, 1941]. Increased pressure in the subarachnoid space in this area may affect both the olfactory fibres crossing it and the surface layers of the olfactory lobe itself. That destruction of nerve fibres on the surface of the olfactory lobe does occur is indeed evident from the fact that in some of the experiments myelin degeneration has been observed in this position. The superficial layer of the olfactory lobe of the brain is often thinner in -A animals, so that the glomerular layer of cells is nearer the brain surface (Figs. 2*b*, 3*b*). This again may be partly due to compression associated with increased intracranial pressure, but the more important factor is doubtless

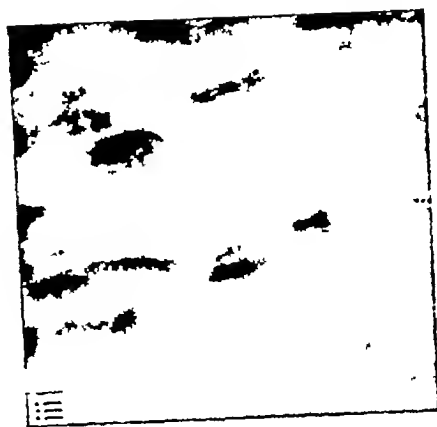


Fig. 1a.

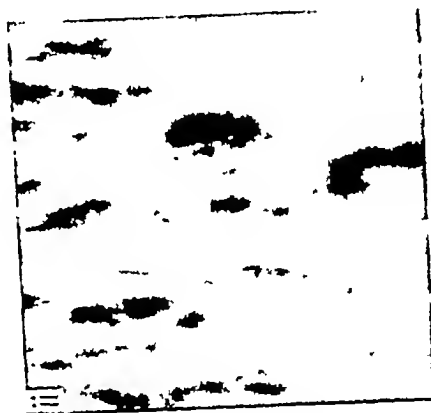
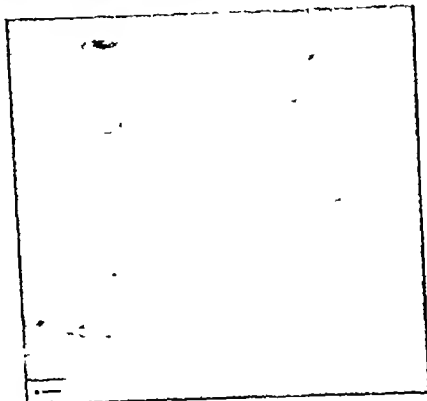


Fig. 1b.



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*, IIInd nerve (optic)*

Reference has previously been made to the degenerative changes in the optic nerve and retina in  $-A$  puppies [Mellanby, 1934*a*] and to the pressure effect of the bone overgrowth surrounding this nerve [Mellanby, 1938]. Only in one of the animals has the optic nerve been completely destroyed, and that animal was on an  $A$ -deficient diet for several years [Mellanby, 1934*a*], a much longer time than the experimental period of the animals examined in the present work, which was usually not over 6 months. Moore, Huffman & Duncan [1935*a, b*] described in calves blindness of a nutritional type resulting from atrophy of the optic nerve where it passes through the optic foramen. This atrophy, they suggested, was caused by improper development of the foramen, and was probably due to bone pressure. They thought that vitamin A deficiency might or might not be the nutritional factor concerned and noted that, while corn silage, timothy hay and cod-liver oil prevented the blindness, 10,000 units of vitamin A ('caritol') did not prevent it. The authors did not notice any bone exostosis but said that 'the bony canal gave more the appearance of having had pressure applied from above, which caused it to become smaller as growth proceeded'. Moore [1939] later, however, came to the conclusion that carotene deficiency was the cause of nyctalopia, papillary oedema and permanent blindness in calves and, although he again ascribed the nerve degeneration to bone pressure due to stenosis of the bony canal, he still thought it was 'difficult to associate vitamin A deficiency with any bony malformation', and that increased intracranial pressure probably accounted for the abnormal bone development.

Evidence will now be given of the manner in which the optic nerve may suffer compression and stretching by bone overgrowth when animals are brought up on diets deficient in vitamin A and carotene. In advanced cases of  $A$  deficiency in dogs, the bone surrounding the optic foramen can be seen from the inside of the skull to be gripping the nerve more closely than usual. Whereas in a normal ( $+A$ ) dog the optic nerve, as it passes through the foramen, lies in loose connective tissue, in a  $-A$  animal it is closely surrounded by bone with but little intervening connective tissue, and in some cases the nerve is gripped tightly and compressed by these tissues. Another possible cause of deformity in the case of the optic nerve, as it passes from the optic foramen into the skull cavity, is pressure from the dura mater which closely surrounds it at this spot; the dura mater in these  $-A$  animals is usually thickened and, possibly because it does not easily adapt itself to the shape of the locally hypertrophied bone, it may be in a state of increased tension at points where nerves pass through it.

Bone overgrowth around the optic nerve in a  $-A$  dog can be clearly seen by comparing serial sections of the nerve in its course from the orbit to the chiasma in  $+A$  and  $-A$  dogs. Fig. 4*a, b* are photomicrographs and Fig. 5*a, b* drawings of sections through the optic nerves of two dogs in the region of the

the disappearance of many olfactory nerve fibres running into the brain in this position.

Histological examinations so far made have not indicated to what extent the normal function of the olfactory nerves in  $-A$  animals is affected. Individual nerve bundles are certainly seen to be compressed in their passage through the cribriform plate, and nearer the brain the number of actual nerve fibres passing across the subarachnoid space seems to be greatly reduced. On the other hand, those fibres that can be seen appear to be normal, except for some degeneration on the surface of the olfactory lobe.

The epithelial or sustentacular cells of the olfactory mucous membrane do not show any great changes in  $-A$  animals, although the cell layers may become irregular in appearance and the nuclei are sometimes found nearer the surface than in  $+A$  animals. The cells remain columnar in shape and do not become squamous or keratinized. The bipolar olfactory nerve cells in the mucous membrane with their hairlets may be reduced in number, but as yet no definite change in individual cells has been observed. As the relative number of receptor cells is known to vary in different parts of the olfactory mucous membrane, even the question of reduced numbers is not certain.

The behaviour of the animals and the histological appearance of the olfactory apparatus above described suggest that the effect of feeding on  $-A$  diets is to reduce both the sense of smell and the number of olfactory nerve fibres. Animals so fed are apt to run about with their noses near the ground sniffing vigorously, as if they were attempting to make up for their deficient smelling power by excessive sniffing. This is the behaviour that might be expected in dogs whose sense of smell, normally well developed, is impaired.

Fig. 3 (a and b). Drawings to explain Fig. 2 (a and b), showing the passage of the olfactory nerve through the cribriform plate. (See also p. 409). (a) Dog whose diet contained vitamin A. (b) Dog whose diet was deficient in vitamin A.

Note: (1) swelling of bones in (b) with enlargement of marrow cavities (Mar.); (2) constriction of foramina of cribriform plate causing compression of olfactory nerve bundles (N) in (b) ( $-A$  dog) as compared with (a) ( $+A$  dog); (3) dura mater thickened and subarachnoid space larger in (b) than in (a); fewer nerve fibres cross the subarachnoid space in the vitamin A-deficient animal (b); (4) outer layers of olfactory lobe thinner in (b) than in (a).

Black represents calcified areas of bone.

Fig. 5 (a and b). Drawings to explain Fig. 4 (a and b), optic nerve near orbit. (See also p. 413). (a) Dog whose diet contained vitamin A. (b) Dog whose diet was deficient in vitamin A.

Note: In (a) both openings of the bony canal through which the optic nerve passes from the orbit to the chiasma can be seen; in (b) the nerve is almost surrounded by bone and, owing to the lengthening and twisting of the canal by the bone overgrowth, only one opening of it is evident.

Black represents calcified areas of bone. Diagonally shaded areas = various portions of IIIrd, IVth, Vth and VIth nerves. E.M. and similarly shaded areas = eye muscles.



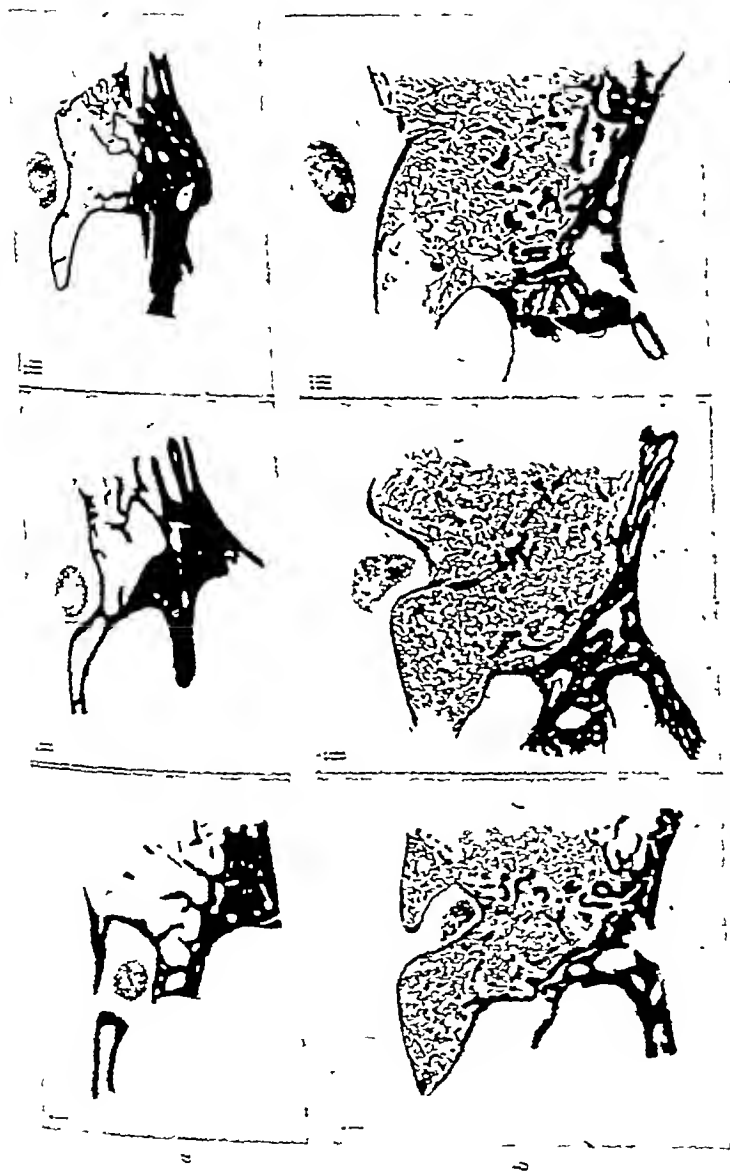


Fig. 6 (a and b) Drawings of sections of three regions of the optic nerve between the orbit and the optic chiasm in (a) vitamin A and (b) -vitamin A dogs (See p. 414). (a) Dog whose diet contained vitamin A. (b) Dog whose diet was deficient in vitamin A.

Note: In (b) grossly enlarged calcified bone as compared with (a) and alteration in shape of the nerve due to compression. Sections (a (i, ii and iii)) are as nearly comparable in position as possible to (b (i, ii and iii)). Black represents calcified areas of bone.

canal formed by the sphenoid bone just as the nerve leaves the orbit on its way to the brain. In the -A dog (Figs. 4 *b*, 5 *b*) the fatty marrow spaces are enlarged, thus increasing the bulk of the bone, which almost completely surrounds the nerve in this position, leaving only a small opening into the orbital cavity and none into the cranial cavity. In the corresponding section of the +A animal (Figs. 4 *a*, 5 *a*) the opening into the orbital cavity is much wider and the optic foramen into the skull cavity is seen. The connective tissue surrounding the nerve is loose in the +A animal, whereas in the -A animal it is tightly packed. That there is some compression of the nerve in the -A animal (Figs. 4 *b*, 5 *b*) at this point is indicated by the fact that: (1) the cross-section of the nerve is oval and not round, as in the +A animal (Figs. 4 *a*, 5 *a*); and (2) the blood vessels in the connective tissue are smaller than in the +A animal. It may be added that, owing to the bone overgrowth described, the optic foramen in the -A animal is longer and more tortuous than that in the +A animal and there is a corresponding increase in length of the nerve.

The appearance of the optic nerve as it emerges from the optic foramen and passes over the surface of the base of the skull towards the optic chiasma can be seen in drawings of serial sections (Fig. 6 *a*, *b*). The bone enlargement in the -A animal (Fig. 6 *b*) again stands out prominently and is seen to be due to the increase in cancellous tissue. The compact bone is not as abundant as in the normal animal (Fig. 6 *a*), so that the total calcified bone of the -A animal is not nearly as great as its increased mass would suggest. It will be noticed that, whereas the optic nerve in the normal animal runs along a gentle depression in the sphenoid bone towards the optic chiasma and is round in cross-section (Fig. 6 *a* (ii, iii)), the depression is much deeper in the -A animal and the nerve is compressed, as can be seen by the distorted shape of its cross section (Fig. 6 *b* (ii, iii)). These are the main bone changes which appear directly to affect the optic nerve. Although there are changes in the anterior clinoid processes of the sphenoid bone, they probably have a greater compressor effect on the pituitary body than on the optic chiasma.

Besides the direct mechanical squeezing of the optic nerve by the overgrown bone, it is probable that the raised intracranial pressure [Mellanby, 1939*a*; Moore & Sykes, 1940] which occurs when there is great bone overgrowth, especially round the posterior fossa, must also be a factor of destruction.

Clinical experience in man teaches that the optic nerve is particularly susceptible to mechanical pressure in any part of its course, and when a nerve fibre is thus injured degenerative changes do not follow the Wallerian law, but the whole neuron may be destroyed. It would be expected, therefore, that in these -A dogs the bone overgrowth which has been shown to exert undue pressure on the optic nerve during its bony passage, as well as the raised intracranial pressure acting on the nerve within the cranial cavity, would have a destructive effect on the optic nerve.

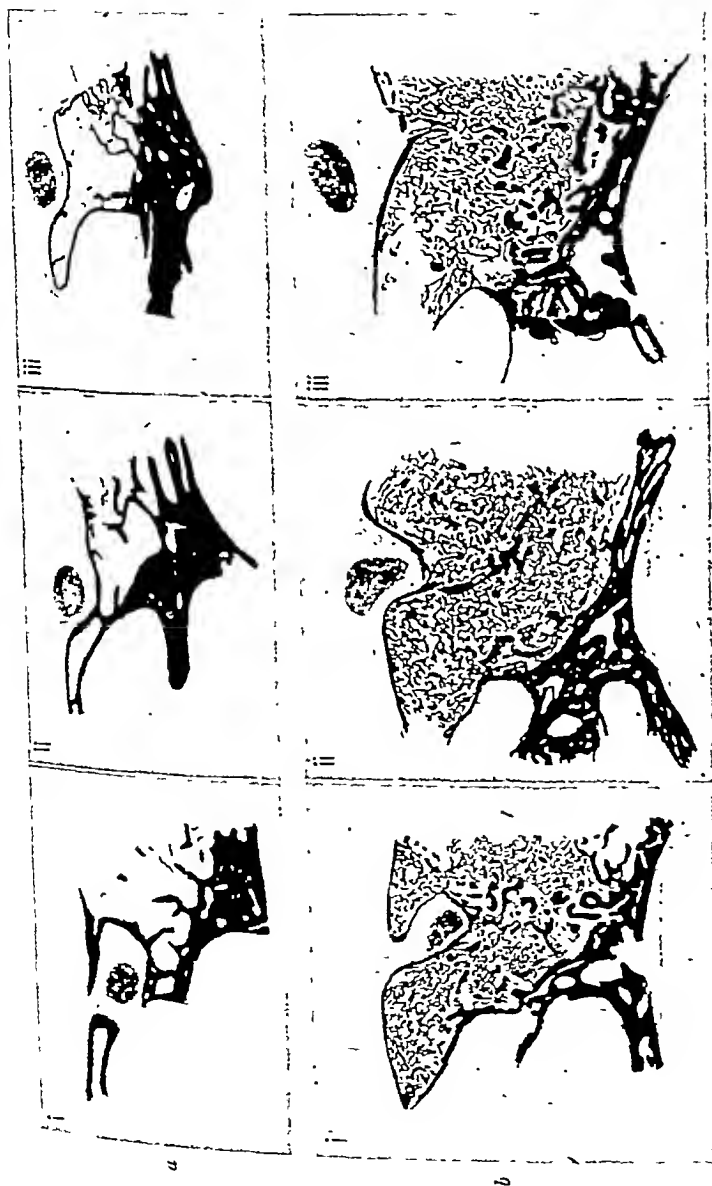


Fig. 6 (*a* and *b*). Drawings of sections of three regions of the optic nerve between the orbit and the optic chiasma in +vitamin A and -vitamin A dogs. (See p. 414.) (*a*) Dog whose diet contained vitamin A. (*b*) Dog whose diet was deficient in vitamin A.

*Note:* In (*b*) greatly enlarged cancellous bone as compared with (*a*) and alteration in shape of the nerve due to compression. Sections (*a* (i, ii and iii)) are as nearly comparable in position as possible to (*b* (i, ii and iii)).

Black represents calcified areas of bone.

Are the pressure effects sufficient to account for the abnormal and degenerative changes that can be observed in —A dogs? The probability is that they are not and that another factor must be taken into consideration, namely, a degenerative change which begins in the retina itself. It is already established that vitamin A deficiency impairs retinal function, as is evident in loss of night vision in man and animals. This loss of function is associated with an upset of the mechanism responsible for the production of visual purple. Since this mechanism begins to fail even in adults after only a few weeks' deprivation of vitamin A, it cannot be associated with any bone hyperplasia or pressure on the nerve, but must be a direct effect of abnormal metabolism on the retinal cells. In dogs other retinal changes develop later, such as degeneration of the ganglion cells which show eccentricity of the nuclei, powdery Nissl granules and reduction in number. The bipolar cells (inner nuclear layer) are also reduced in number and lose some of their affinity for basic dyes [Mellanby, 1934*a*]. Ultimately, the whole of the ganglion cells of the retina and the neurons of the optic nerve may disappear, but this degree of degeneration is only reached after many months of A deficiency. It is, of course, probable that many of the retinal and nerve changes, especially the later changes, may be produced by pressure of overgrown bone on the optic nerve. On the other hand, some of the degeneration may be an extension of the retinal defect of night blindness produced directly by vitamin A deficiency. Is there any evidence of this?

In 1939 Moore pointed out that papilloedema and bleaching of the tapetum could be observed in vitamin A-deficient calves by ophthalmoscopic examination. Similar changes are found in —A dogs, but there is reason to believe that the condition of the optic disk is not simply one of papilloedema. The earliest retinal change observed in these animals by ophthalmoscope is an alteration in the colour of the tapetum lucidum, which loses its blue component. The blue band contiguous with the tapetum nigrum becomes green and the green coloration gradually changes to yellow from its upper boundary downwards to the tapetum nigrum. Ultimately, the whole of the tapetum lucidum is yellow and the tapetum nigrum in some of the —A dogs' eyes seems rather a darker brown than in the normal dogs.

The early changes in the tapetum are soon followed by pallor of the disk, which may be associated with protrusion of about half a diopter. Swelling of the optic disk to a degree which can be described as definite papilloedema takes place slowly after these initial changes, and in some of the dogs reaches as much as 8 diopters. In the early stages the pale disk, when viewed with the ophthalmoscope, has a sharp outline. Later the edge is blurred and irregular, in some cases with apparent extensions of the disk along the vessels, especially on the nasal side.

An attempt was made in the course of the work to see whether there was any

relationship between the degree of abnormality of the optic disk and the degree to which overgrown bone compressed the nerve as it entered the cranial cavity from the optic canal, but the evidence was inconclusive. This may possibly be regarded as supporting the view that a factor other than pressure of bone and raised intracranial pressure is involved. This other factor is probably the direct action of vitamin A deficiency on the retinal cells which begins to manifest itself early in the experimental period by bleaching of the tapetum lucidum and pallor of the disk. At a later stage bone pressure on the optic nerve and the increased intracranial pressure become effective and produce a condition of papilloedema. While, therefore, it cannot be said that the position is yet clarified, ophthalmoscopic and histological evidence suggest that the optic nerve degeneration in  $-A$  animals is really a double mechanism, one a condition of optic atrophy starting in the retina itself, and the second a condition of papilloedema superimposed on the atrophy by the pressure of bone on the optic nerve and by the increased intracranial pressure.

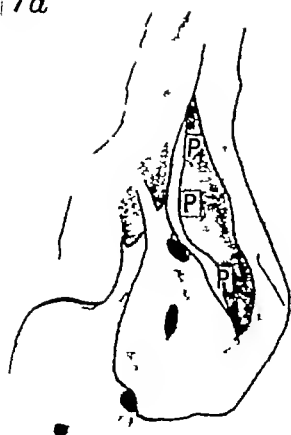
*IIIrd, IVth and Vth nerves (oculomotor, trochlear and abducens)*

These nerves, together with the first branch of the Vth, pass through the superior orbital fissure. There appears to be but little bone change in this region; at least, there is not sufficient change to compress the nerves to any great extent; and the only nerve passing through this fissure which is regularly found to be degenerated is the first branch of the Vth, which will be considered later with the trigeminal system. Allusion has been made above to the distorting effect of the dura mater on the optic nerve. A similar effect can sometimes be seen on the IIIrd nerve of  $-A$  dogs. This nerve is normally slightly bent at the point where it passes through the dura mater into the cavernous sinus, but in  $-A$  dogs the distortion is exaggerated. It may be noted that in the one case in which the region of this distortion was examined histologically, a few degenerated fibres were found. Since, however, there is seldom much degeneration in the IIIrd nerve in  $-A$  animals, and indeed this applies to all the motor nerves of the eye, the mechanical distortion produced in this region by the dura mater alone does not apparently interfere to any great extent with the structure of the nerve fibres or their function.

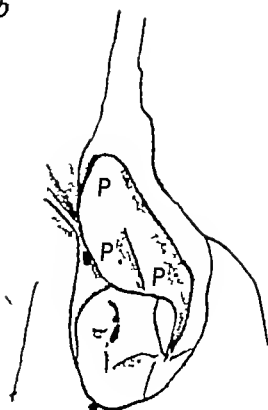
*Vth nerve (trigeminal)*

Degenerative changes in the Vth nerve and in the Gasserian ganglion in  $-A$  animals have been previously described [Mellanby, 1934b]. It was shown that there was often an association between degeneration of the fibres of the first division of the Vth nerve and xerophthalmia, and it was suggested that this latter condition might be a manifestation of loss of neurotrophic control of the nerve over the conjunctiva and cornea. Whether this is so or not, the two conditions, xerophthalmia and degeneration of the sensory branches of the Vth

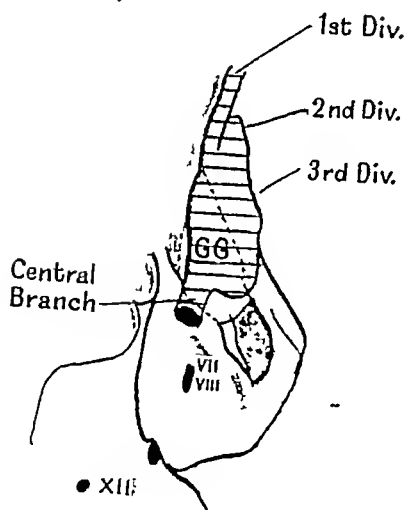
7a



7b



8a



8b

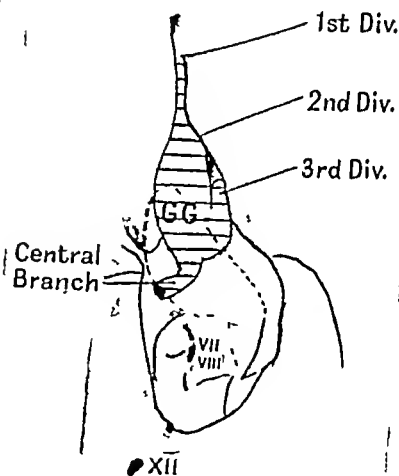


Fig 7 (a and b) Drawings of the petrous ridge (P) of temporal bone of +vitamin A and -vitamin A dogs (See p 419) (a) Dog whose diet contained vitamin A (b) Dog whose diet was deficient in vitamin A

Note Thickened bulbous ridge in (b) as compared with (a)

Fig. 8 (a and b) Drawings of the same specimens as Fig 7 (a and b) after removal of the petrous ridge to expose the Vth nerve and Gasserian ganglion (GG) (a) Dog whose diet contained vitamin A (b) Dog whose diet was deficient in vitamin A

Note Bone overgrowth in (b) has twisted the central branch of the Vth nerve, in (a) this branch is straight and normal Note also the reduced size and folded wall of the VIIIth nerve foramen in (b)

nerve, are generally found together in dogs, rabbits and rats. M. Mellanby & King have shown in -A animals degeneration of Vth nerve fibres supplying the gums and teeth, together with hyperplasia of the gum epithelium [Mellanby & King, 1934] and changes in the dental roots [King, 1936]. There is therefore no doubt about the destruction of sensory fibres of the Vth nerve in -A animals, and it now remains to see whether the bone changes at the base of the skull in the neighbourhood of the nerve are sufficient to account for this degeneration.

Macroscopic examination of the skulls of -A dogs reveals at once large overgrowth of the bone surrounding the Vth nerve system as it passes under the petrous ridge of the temporal bone. The petrous ridge is swollen and blunted, the increase in size being in depth and width rather than in length, as can be seen in Fig. 7*b* (cf. Fig. 7*a*, the normal). When the bone of the petrous ridge is removed (Fig. 8*a, b*), it will be seen that the bone overgrowth has greatly affected the course of the Vth nerve. In the -A animal (Fig. 8*b*) the central branch of the nerve is bent and it passes from the bony foramen towards the pons in a more mesial position. In the normal animal (Fig. 8*a*) the central branch of the nerve runs straight from the Gasserian ganglion to the pons without any bending.

Fig. 9*a, b* shows drawings of sections, as far as possible comparable, through the trigeminal nerve and petrous bone of a +A and a -A dog respectively. Whereas in the +A animal (Fig. 9*a* (i, ii)) the nerve is cut transversely to its length and occupies a relatively wide space in the bone, the twisting of the nerve in the -A animal is such that it is seen to be cut parallel to the plane of the section (Fig. 9*b* (i, ii)). The overgrowth of the petrous ridge has also encroached on the space occupied by the nerve and has compressed it. A small portion of bone (X) can be seen immediately beneath the nerve in Fig. 9*a* (i, ii), but in the -A animal (Fig. 9*b* (i, ii and iii)) this piece of bone (X') is much greater in size and, together with the overgrowth of the petrous ridge, has caused considerable compression of the Vth nerve. Fig. 9*a* (vi) shows the normal appearance of the Gasserian ganglion in relation to the bone surrounding it. In Fig. 9*b* (vi), a drawing of a section through the same region in a -A animal, the Gasserian ganglion is compressed and elongated by the great overgrowth of the petrous portion of the temporal bone. It will also be seen that the depression of the internal surface of the petrous ridge into which the ganglion fits (Fig. 9*a* (vi)) has become flat in the -A animal (Fig. 9*b* (vi)). Although the Gasserian ganglion is squeezed between the apex of the petrosal portion of the temporal bone and its petrous ridge, the nerve cells do not show the elongation that might be expected; they do, however, undergo chromolytic and other degenerative changes [E. Mellanby, 1934*b*], which may be due to the mechanical pressure of the abnormal bone.

In cases where the effects of the A-deficient diet are slighter, the overgrowth of the petrous portion of the temporal bone may not be so obvious and twisting



Fig. 9 a.



Fig. 9 b.

Fig. 9 (a and b). Drawings of serial sections through the Vth nerve, Gasserian ganglion and surrounding bone in + vitamin A and - vitamin A dogs. (See p. 419.) (a) Dog whose diet contained vitamin A. (b) Dog whose diet was deficient in vitamin A.

Note: (1) overgrown petrous ridge in (b) as compared with (a); (2) in (b (i, ii and iii)) the nerve is cut longitudinally, whereas in (a (i, ii and iii)) it is cut transversely; this is due to twisting of the nerve in (b) similar to that shown in Fig. 8 (b); (3) in (b (v)) the ganglion can be seen to be compressed between the petrous ridge and the apex of the petrous bone, as compared with the ganglion in (a (v)).

Black represents calcified areas of bone.

X and X'. For explanation see text.



the central branch of the Vth nerve may not occur. Fig. 10*b* is a section through the foramen lacerum of such a dog and compares with a section from control dog of the same age (Fig. 10*a*). The overgrowth of bone in the -A animal is again obvious, the additional bulk being made up of loose cancellous bone at the expense of the compact bone seen in the control animal (Fig. 10*a*). It will be seen also in Fig. 10*b* how deeply placed the nerve is, due to the bone hypertrophy. The deformity of the nerve in Fig. 10*b* is obvious; it is now

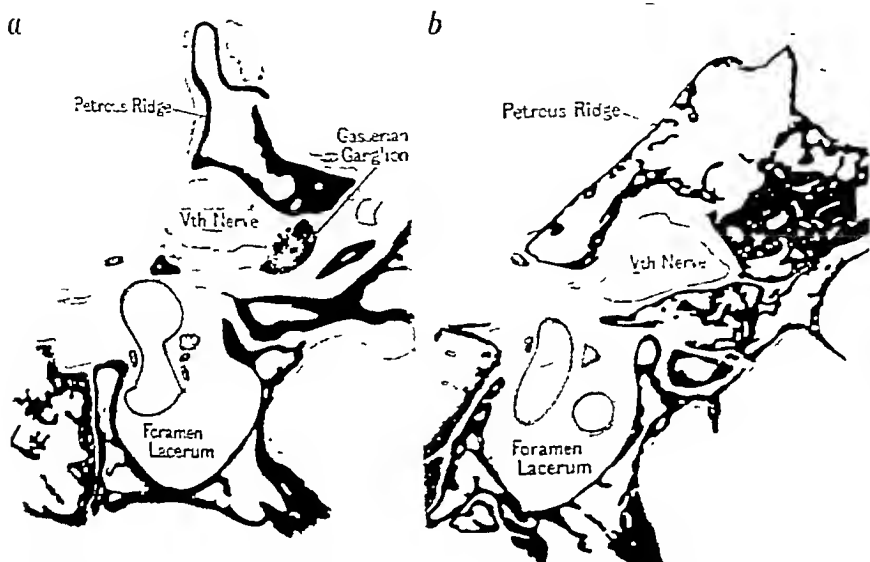


Fig. 10 (a and b). Drawings of sections through the Vth nerve and surrounding bone at the level of the foramen lacerum in +vitamin A and -vitamin A dogs. (a) Dog whose diet contained vitamin A. (b) Dog whose diet was deficient in vitamin A.

Note: In (a) the nerve and part of the ganglion are surrounded by loose tissues, whereas in (b) no ganglion cells are seen and the nerve is almost surrounded by overgrown and encroaching bone which has altered its shape.

Black represents calcified areas of bone.

triangular in cross-section to conform to the shape of the bony canal (cf. Fig. 10*a*), and this change of shape again indicates a mechanical effect of the bone on the nerve during its passage to the brain from the tissues outside the skull.

When the Vth nerve has emerged from under the petrous ridge in its passage towards the periphery, it assumes a more normal appearance, since the bone overgrowth produced by the vitamin A-deficient diets is less prominent, although still definite. For instance, if one examines sections passing through the foramen ovale, the sphenoid bone is seen to be thickened and the foramen is narrowed, but not sufficiently to press on the third (mandibular) branch of

the Vth nerve and distort its shape. The sphenoid bone surrounding the foramen rotundum, through which the second (maxillary) branch of the Vth nerve passes, is also slightly thickened and the foramen narrowed, but again there is no evidence of bone pressure on the nerve.

The first (ophthalmic) branch of the Vth nerve, together with the IIIrd, IVth and VIth nerves, passes through the superior orbital fissure towards the orbit. The bone is thickened in this position, but not so as to reduce the fissure and there is no evidence of bone pressure on the nerves traversing it. It is possible that the nerves in this position are affected by increased intracranial pressure, suggestive evidence of which is obtained by examining the blood vessels running with the nerves. In the cases examined they are smaller than the corresponding vessels in the same position in normal animals. The connective tissue surrounding the nerves and vessels seems also to be compressed and not only to occupy a smaller space but to be denser in the vitamin A deficient dogs than in the control animals. This possible effect of increased pressure on blood vessels in the brain may be of importance and requires more detailed study.

One other mechanical effect of bone overgrowth which may be of some importance, is the increase in length of the Vth nerve. This lengthening becomes more apparent on cutting serial sections of the nerve and its surrounding bone in comparable +A and -A dogs. It may involve actual stretching, or the nerve may simply lengthen without any increase in tension. It was thought at first that the lengthening of the nerve might itself produce degeneration, but a similar lengthening produced by thickening of bone was found to occur in other cranial nerves, without comparable degenerative changes. This applies especially to the IXth, Xth, XIth and XIIth and, since little or no degeneration is found in these nerves in -A animals, it is probable that the degeneration suffered by Vth nerve is not due to lengthening.

It seems from the foregoing account that the great susceptibility to destructive change of the more purely sensory divisions of the Vth nerve in -A dogs could be accounted for by bone overgrowth pressing on the Gasserian ganglion.

#### *VIIth nerve (facial)*

It has been shown in earlier publications that the motor cranial nerves are relatively little damaged in -A animals in which the sensory cranial nerves are severely affected [Mellanby, 1935]. Of the motor cranial nerves, probably the VIIth is more often affected by degenerative changes whilst, among the sensory cranial nerves, the adjacent VIIIth is the most often destroyed. On the other hand, there is a great difference between the liability of neurons of the VIIth and VIIIth nerves to destructive changes, due probably in large measure to the different positions of their cells of origin.

In its passage from the central nervous system to its exit from the skull, the VIIth nerve may be roughly divided into five parts: (1) the portion from the point of issue from the brain stem to the internal auditory meatus; (2) the portion running close to the VIIIth nerve within the internal auditory meatus; (3) a short portion which leaves the internal auditory meatus near the modiolus and connects with the geniculate ganglion; the nerve here is within the facial canal which passes in an antero-lateral direction; (4) a part which, beginning at the geniculate ganglion, bends through an angle of practically  $120^\circ$  and then passes posteriorly through the facial canal in the upper wall of the tympanic cavity; and (5) the portion which passes through the stylomastoid foramen.

Taking these parts separately, it is obvious that the first part of the VIIth nerve running from the brain stem to the internal auditory meatus will not be affected directly by bone overgrowth and, in fact, it will only be subjected to the same increase of intracranial pressure as may influence any other part of the central nervous system in these — A animals. The second part of the nerve will be subjected to the same conditions as the VIIIth nerve in this position. Bone overgrowth in the neighbourhood of this part of the course of the VIIIth nerve in — A animals has been described in an earlier publication [E. Mellanby, 1938]. The internal auditory meatus is often greatly lengthened and twisted by overgrown periosteal bone of the labyrinthine capsule, so that the passage may in individual sections seem to be occluded, but if a series of sections is examined it is found that the meatus is constricted but patent. If there is ever complete destruction of the VIIth nerve due to bone overgrowth at this part of its passage, it must be very rare. Nor is it this particular bone overgrowth around the internal auditory meatus which is responsible for the severe degeneration of the cochlear division of the VIIIth nerve. The new bone mainly responsible for this change is found at the modiolus end of the internal auditory meatus adjacent to the helix which contains the ganglia of the auditory division. These ganglion cells, as previously described [Mellanby, 1938], are often destroyed and the whole neuron may be killed by bone pressure. The VIIth nerve, however, does not reach the modiolus, but turns away from the VIIIth nerve to enter the facial canal some distance before the helix is reached. It would appear, therefore, that although the VIIth nerve, when passing through the internal auditory meatus, is liable to suffer some stretching and compression by overgrowth of periosteal bone, there is no evidence that it suffers severe destructive changes in this position.

In the third part of its course through the facial canal, the VIIth nerve is again liable to be compressed by the partial closure of the canal. Layers of new-formed bone can be seen lining the canal in — A animals. The resultant narrowing of the canal does not proceed to complete occlusion, but it may be sufficiently great to press on the geniculate ganglion and elongate the cells in a way similar to, but to a less degree than, that seen in the posterior root ganglion

and in Scarpa's ganglion in — A dogs [Mellanby, 1938]. In spite of the elongation by pressure, the cells of the geniculate ganglion, from which the sensory fibres of the VIIth originate, suffer but little destructive change, and their Nissl's granules and nuclei generally seem normal or nearly so. In severe cases, however, there is definite destruction of some of the cells. It is obvious from the appearance of the cells and the canal in such cases that the cells can withstand a good deal of pressure and distortion without degenerating. It may be that, when degenerating fibres have been found in the VIIth nerve, they are fibres having their cells of origin in the geniculate ganglion.

In the fourth part of its course, where the VIIth nerve runs through the facial canal in the upper wall of the tympanic cavity, the compression due to bone overgrowth may be severe. In several cases the nerve has been seen to be compressed to a thin ribbon, but complete occlusion of the canal has never been observed even in the most severely affected cases; nor, indeed, have the blood vessels which pass with this part of the nerve appeared unduly narrowed.

The fifth part of the nerve now issuing from the skull wall through the stylo-mastoid foramen is unrestricted by bone overgrowth and the passage usually appears to be quite normal.

It seems, therefore, that the VIIth nerve is liable to be affected by bone overgrowth in the second, third and fourth parts of its course, and it is in these positions that degenerating nerve fibres may sometimes be found. Even in severe cases of A deficiency, however, the number of such fibres is relatively small.

Reference may be made here to two nerves which pass into the VIIth nerve, namely, the greater superficial petrosal nerve going from the spheno-palatine ganglion on the second division of the Vth nerve to the geniculate ganglion on the VIIth nerve and the auricular nerve which passes from the jugular ganglion of the Xth nerve to join the VIIth nerve near the stylomastoid foramen. Both these nerves may be compressed in — A animals, but especially the greater superficial petrosal nerve which, in advanced cases, may be pressed to a ribbon shape by the encroaching bone. In spite of this distortion, however, there may be only a few fibres in the nerve showing degenerative changes. The compression of the auricular branch of the Xth nerve is not so great, and here again the nerve usually shows only a few degenerated fibres.

Facial paralysis has not been recognized in the animals used in these experiments and, in spite of the severe constriction of the nerve, sufficient degeneration to justify paralysis has not been found. In human beings also the VIIth nerve is known to be very resistant to pressure. The absence of facial paralysis in dogs and man in spite of compressed VIIth nerves, emphasizes the fact that some nerve fibres can withstand a surprising amount of mechanical pressure and distortion without loss of function.

*IXth, Xth and XIth nerves (glosso-pharyngeal, vagus and accessory)*

These nerves pass together from the cranial cavity through the jugular canal, the walls of which are formed by portions of the temporal and occipital bones. Both these bones are thickened in -A dogs, but in spite of this, the jugular canal is only slightly narrowed, the main effect being the lengthening of the canal from the base of the brain to the wall of the tympanic cavity. At this latter point the jugular canal turns slightly and passes over (round) the wall of the tympanic cavity. It might be expected that, in passing over the tympanic cavity and under the thickened basi-occipital bone, the nerves would be subjected to increased pressure. This, however, does not appear to be the case. It seems probable that constriction of the canal is avoided at this point because the space occupied by the thickened occipital bone is obtained at the expense of the tympanic cavity which is smaller and surrounded by thicker walls than in the +A animals.

The only change in diameter of the canal appears to be near the internal end. Here the bone is sometimes seen to be folded and the ganglia on the nerves seem to fit more tightly in the canal. Changes in these ganglia when examined either by naked eye or histologically are very slight, even in severe vitamin A deficiency. Generally speaking, the only mechanical change observable in these animals is the lengthening of the nerves owing to bone hypertrophy, and there is no conspicuous pressure of bone on either the nerves or their ganglia. Examination of the nerves by Marchi's method shows that very few degenerated fibres are present in those parts which are within the jugular canal. After the nerves have emerged from the canal a few more degenerated fibres can be

One outstanding fact is that under the conditions of these experiments the sensory nerves are largely damaged, while the motor nerves generally escape destructive changes. It has been found that the VIIIth nerve (both divisions, but especially the cochlear division), the sensory fibres of the Vth (all divisions), the optic nerve and the olfactory nerve are partially destroyed. The IIIrd, IVth, third branch of the Vth, VIth, VIIth, IXth, Xth, XIth and XIIth tend to escape destructive changes.

In considering this problem the following factors must be taken into account: (a) direct pressure of overgrown bone on nerve cells and fibres, (b) the restriction of blood supply to nerve cells and fibres by the overgrowth of bone, (c) an increased intracranial pressure, again due to overgrowth of bone, (d) a direct effect of vitamin A deficiency on certain nerve cells. There is little doubt that the direct pressure effect on nerve cells and fibres is the main cause of nerve degeneration in these animals, but it is still far from clear what part the other factors may play.

It is probable that the chief reason for the difference between sensory and motor nerve susceptibility to damage is the presence in the course of the sensory nerves of ganglia outside the central nervous system and the pressure to which they are subjected by overgrown bone. Motor nerves, having their ganglionic origin within the central nervous system, escape this form of pressure, and only the nerve axons can be squeezed by direct bone overgrowth. Of the sensory-nerve systems, the Gasserian ganglion of the trigeminal nerve is liable to be compressed by the overgrowth of the petrous bone, so that sensory fibres of all three divisions show large degenerative changes. The third (mandibular) division contains a much smaller proportion of degenerated fibres than the second (maxillary) or the first (ophthalmic) division. The motor fibres of the third division apparently escape destructive changes, for, although their axons run with the Gasserian ganglion through the petrous bone and suffer the same pressure changes, their cells of origin are inside the central nervous system.

In the case of the optic nerve, a further instance must be considered of the explanation offered in 1938 [Mellanby] to account for the differences in susceptibility of the cochlear and vestibular divisions of the VIIIth nerve to destructive changes in vitamin A-deficient animals. In that publication the evidence was given to show that, whereas pressure on any part of the neuron of the cochlear division caused complete degeneration, similar pressure on the vestibular neuron only caused degeneration expected by the Wallerian law, namely of that part of the neuron peripheral to the injury. Clinical experience in man shows that pressure on any part of the optic nerve causes degeneration both of the nerve and of its ganglion cells in the retina.

It seems established, therefore, that, apart from the degree of pressure produced on nerves by local bone overgrowth and apart from the exposure of the ganglia of certain cranial nerves to such pressure, the greater degree of

susceptibility of some cranial nerves than of others to injury must also be a factor in determining the amount of degeneration produced in animals having vitamin A-deficient diets. This variation in susceptibility to injury, while clearly of importance in determining the degree of degeneration in the sensory nerves of the brain, may well also partly account for the relative immunity of the motor nerves to degeneration. Thus, whereas many fibres of the olfactory nerve are killed by bone pressure in their passage through the overgrown cribriform plate in vitamin A-deficient animals, the facial nerve often suffers great deformity by pressure in the same animals without showing any destruction. The same resistance to destruction shown by the facial nerve has been observed clinically in man, and Perlman & Willard [1941] have pointed out that this nerve 'may be compressed to a microscopically thin ribbon on the capsule of the tumour and yet no facial paralysis is seen'. It may be therefore that, even excluding the auditory division of the VIIIth nerve and the optic nerve because of their exceptional susceptibility to injury, motor nerve axons escape destructive changes when exposed to those pressure effects which would destroy the axons of sensory nerves. On the whole, however, in the present experiments the motor nerves, except the motor fibres of the Vth and the facial nerve, escape pressure changes due to bone hypertrophy. Some of them, especially the IXth, Xth, XIth and XIIth, may be lengthened owing to the increased thickness of the temporal and occipital bones in vitamin A-deficient dogs, but this change does not cause degeneration. It seems probable that these nerves are never seriously stretched but adapt themselves by growing in length as the bone increases in thickness.

Attention has already been called to the increased susceptibility of the optic nerve to injury, and at one time it was thought that this susceptibility accounted for the fact that the optic nerve showed more degeneration than would be expected from the degree of bone pressure it experienced in these animals. Some doubt has arisen about this recently because of the early retinal changes that develop in -A animals, and it is possible that a deficiency of this vitamin has a degenerative effect on the ganglion cells of the retina which is independent of pressure on its nerve fibres. The point has been discussed above, and evidence was given which suggested that the optic nerve in -A animals suffered two kinds of destructive change: (1) an early condition of optic atrophy probably beginning in the retina itself, and (2) papilloedema superimposed on the optic atrophy and due to pressure on the nerve by the neighbouring overgrown bone together with the increased intracranial pressure. If it should prove that the retinal cells are directly influenced by vitamin A deficiency and that optic atrophy is produced independently of bone overgrowth and pressure, the problem reverts partially at least to its earlier position where it was considered that the abnormal metabolism associated with vitamin A deficiency might itself cause degeneration of certain nerve ganglia [Mellanby, 1934b].

It raises the possibility once more that, in addition to the destructive nerve changes due to bone and intracranial pressure, there may be other instances of nerve degeneration in the central nervous system which depend directly upon the normal metabolic changes associated with A deficiency.

One factor which has not been considered in this publication is the effect of the bone overgrowth on the blood supply. If, as seems possible in areas of great malformation, the blood supply is interrupted or reduced, the effect on the nutrition of the nerves, and more especially the nerve cells, would be considerable. No instance has been seen of a foramen being so occluded as to obliterate the blood vessels, and it may be that the blood supply remains adequate.

Wolbach & Bessey [1941] have recently given their authority to the view that degenerative changes in nervous tissue in -A animals are easily explained by cessation of bone growth at a time when the nervous system is growing normally. This, they claim, causes compression of the nervous system and subsequent degeneration. They refer to the older observations of Hess, McCann & Pappenheimer [1921] and those of Wolbach & Howe [1925] on cessation of bone growth in -A rats and suggest that this older work suffices to explain the recent results. It may be permissible to draw attention to the fact that the diets used in these older experiments were deficient both in vitamin A and vitamin D. It would be unfortunate if this view of Wolbach & Bessey were accepted, for the evidence indicates that, in vitamin A deficiency, growth of bone does not cease in dogs, rabbits or, in my experience, even in rats.

Wolbach & Bessey [1941] confirm the observations of Mellanby [1938] and of Loch [1939] on the increase in the periosteal bone of the labyrinthine capsule and on the formation of exostosis in the internal auditory meatus in vitamin A-deficient rats, while Perlman & Willard [1941] have reported similar excessive bone growth in rabbits. The same intrusion of cancellous bone into the canal surrounding the optic nerve of calves is shown in a photomicrograph published by Moore, Huffman & Duncan [1935*a*], although they do not comment in this paper on the cancellous bone formation nor relate it to vitamin A or carotene deficiency. There is therefore good evidence that a deficiency of vitamin A and carotene in all young experimental animals tested produces bone overgrowth of a specific kind. There may be some special reason for the experimental results obtained by Wolbach & Bessey in rats, and the conclusion of these authors that bone growth ceases in vitamin A deficiency.

In the investigations described here and elsewhere certain bones of vitamin A-deficient dogs are larger than those of normal animals and they grow in an abnormal way. Some bones grow more than others and some parts of a bone grow more than other parts. Their general normal outline is changed and they become coarser in appearance and lose their fine moulding. In the dog the greater thickness of the malar bone and the zygomatic portion of the temporal



bone can be seen on examining the skull. Similarly, the lower jaw is often greatly thickened. Other instances, e.g. supra- and basi-occipital, and the sphenoid and temporal bones, have been given of excessive bone growth in the sense described.

It is true that some of the internal openings of the cranial foramina in — A dogs are smaller than usual and compress the nerves, and this may at first glance suggest cessation of bone growth at a time when the nerve tissues continue to grow at a normal rate. Further examination of the bone at these points, however, shows that this interpretation is incorrect, for the openings are not circular or oval as in the normal animal, but the walls are folded because of bone overgrowth, thus making the foramina triangular or irregular in shape. In contrast to this, it can sometimes be observed that the external ends of these bony canals are larger than normal.

The evidence indicates that a certain amount of vitamin A is necessary for normal bone growth and that, when there is a deficiency, bone does not stop growing, as suggested by Wolbach and Bessey, but a controlling influence on its growth is lost. The size of the bones continues to increase but some are malformed and contain an excessive amount of cancellous tissue whose spaces are often full of fatty marrow. This overgrowth of cancellous bone may be accompanied by a reduction in compact bone, as is shown in the illustrations above (Fig. 6), but this is not always the case, and if the calcium, phosphorus and vitamin D of the diet are generous, the compact tissue may also be thicker, even if not normal in structure, in some vitamin A-deficient bones. In vitamin A deficiency there is a bone dysplasia. The function of this vitamin in young growing animals is not that of a stimulant to bone growth but rather that of a controller of certain growth elements, the co-ordinated activity of which is necessary for normal bone production. When vitamin A is deficient, the activity of these bone elements is unco-ordinated, the growth becomes excessive in places, and the nervous tissue is compressed and suffers destructive changes. In the adult animal vitamin A acts in a similar way, but since ordinary growth has ceased, the specific effects on bone are much slower in developing and are never as conspicuous as in young animals.

#### SUMMARY

1. When young dogs are brought up on diets deficient in vitamin A and carotene, local overgrowth of certain skull bones causes compression, twisting and lengthening of most cranial nerves, some of which show large degenerative changes. These changes are intensified if, as happens in the VIIIth and Vth nerves, the ganglion cells are also affected by bone overgrowth.

2. Destructive changes are largely confined to the sensory nerves, the motor cranial nerves for the most part escaping.

3. Cranial nerves, especially those with motor function, such as the VIIth, can often suffer compression, lengthening and twisting as the result of bone overgrowth without degenerating.

4. In the experiments described the nerves most affected in diminishing order are somewhat as follows: (a) cochlear and vestibular divisions of the VIIIth nerve, especially the former, (b) Vth nerve (trigeminal) (first and second branches especially), (c) IIth nerve (optic), (d) Ist nerve (olfactory).

5. Degeneration in the optic nerve may be produced in -A animals, not only from direct pressure of overgrown bone and from increased intracranial pressure, but also from a primary degenerative change beginning in the retina itself. The early optic atrophy associated with bleaching of the tapetum is probably a direct effect of A deficiency on retinal cells, while papilloedema, due to bone overgrowth and increased intracranial pressure, is superimposed later.

6. Whereas the internal ends of foramina in the skulls of vitamin A-deficient animals are generally stenosed, with folded outlines due to bone overgrowth and not to cessation of growth, the external openings are not usually smaller than normal.

7. In these experiments, where the calcium intake was not high, the increased bulk of certain bones is due to the formation of an excess of cancellous tissue.

I wish to acknowledge the great help given to me in this work by Mr R. J. C. Stewart.

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## EXPLANATION OF PLATES 1 AND 2

## PLATE 1

Fig. 1 (*a* and *b*). Photomicrographs ( $\times 500$ ) of sections of branches of the olfactory nerves (non-medullated) of +vitamin A and -vitamin A dogs. (*a*) Dog whose diet contained vitamin A. (*b*) Dog whose diet was deficient in vitamin A. (*a* (i)) and (*b* (i)). Before entering the bony plate; note approximately same number of sheath nuclei in each. (*a* (ii)) and (*b* (ii)). Within the bony plate; note many more nuclei in the -A (*b*) than in the +A (*a*) owing to squeezing of the nerve by the bone. (*a* (iii)) and (*b* (iii)). After emerging from the bony plate; note nuclei rather more concentrated in (*b*) than in (*a*), but less concentrated than in *b* (ii). (See p. 410.)

The olfactory nerve of the -A dog has been squeezed in passing through the cribriform plate.

## PLATE 2

Fig. 2 (*a* and *b*). Photomicrographs ( $\times 6$ ) of sections showing olfactory nerves passing through the cribriform plate of +vitamin A and -vitamin A dogs. (See Fig. 3, representing drawings of Fig. 2, for explanation.) (*a*) Dog whose diet contained vitamin A. (*b*) Dog whose diet was deficient in vitamin A.

Fig. 4 (*a* and *b*). Photomicrographs ( $\times 6$ ) of sections of the optic nerve (near orbit) and surrounding tissues of +vitamin A and -vitamin A dogs. (See Fig. 5, representing drawings of Fig. 4, for explanation.) (*a*) Dog whose diet contained vitamin A. (*b*) Dog whose diet was deficient in vitamin A.

SYNTHESIS OF ACETYLCHOLINE IN SYMPATHETIC  
GANGLIA AND CHOLINERGIC NERVESBy W. FELDBERG, *From the Physiological Laboratory,  
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The evidence for the theory of chemical transmission by acetylcholine across ganglionic synapses points to the preganglionic nerve endings as the seat where the acetylcholine is liberated. When released it is quickly replaced by synthesis so that the normal store of acetylcholine does not diminish even after prolonged preganglionic nerve stimulation [Brown & Feldberg, 1936*b*; Kahlson & MacIntosh, 1939]. Thus it appeared likely, as suggested by Brown & Feldberg, that synthesis of acetylcholine is not a property of the cells in the sympathetic ganglia but of the preganglionic endings and, in that case, it may not be confined to the endings but may occur along the whole length of a cholinergic nerve. The experiments described in this paper were carried out to test this conception.

Synthesis of acetylcholine has been described in the central nervous system by Quastel, Tennenbaum & Wheatley [1936] and Mann, Tennenbaum & Quastel [1938, 1939]. Although some of their interpretation is no longer valid [Trethewie 1938; Stedman & Stedman, 1939], they were the first to show that tissue slices, or suspensions of minced brain, synthesize acetylcholine when respiring. Their results have been confirmed by Trethewie [1938]. About the same time Stedman & Stedman [1937, 1939] found that minced or ground brain, incubated in a medium of chloroform or ether, synthesizes acetylcholine. Synthesis in sympathetic ganglia has been observed in the perfused superior cervical ganglion of the cat during prolonged stimulation of the cervical sympathetic [Brown & Feldberg, 1936*b*; Kahlson & MacIntosh, 1939]. No direct experiments have been carried out to demonstrate synthesis in cholinergic nerves, but there are some observations pointing in this direction. Von Murralt [1937] found that a nerve 'shot into liquid air' during stimulation gave an increased yield of acetylcholine on extraction, and Rosenblueth, Lissák & Lanari [1939] observed a similar increase in nerves extracted a few minutes after prolonged stimulation.

## METHODS

We used a modification of the method described by Mann *et al.* [1938] for brain tissue, and studied synthesis of acetylcholine in finely divided tissue from normal sympathetic ganglia, from sympathetic ganglia at different stages of preganglionic nerve degeneration, and from the normal and degenerating cholinergic nerves of sheep and cats.

The sheep were bled under spinal duracain or intravenous nembutal anaesthesia and the ganglia and nerves removed immediately after death. The cats were anaesthetized with ether, followed by intravenous injection of chloralose, the ganglia and nerves being removed from the living animal or immediately after death. In some cats they were removed aseptically under ether anaesthesia. The ganglia of one side were used as controls. In order to obtain comparable samples of nerve tissue, each nerve was cut into pieces about 1 cm. in length, which were used alternatively along the nerve for the two samples. The ganglia and nerves were dried between filter paper, weighed and extracted or incubated.

For the determination of the normal acetylcholine content of the tissue the following procedure of acid extraction with boiling was adopted. Ganglia and nerve samples weighing less than 50 mg. were brought into a solution made up of 0.5 c.c.  $N/3$  HCl, 1 c.c. bicarbonate-free Locke solution, 0.3 c.c. phosphate buffer solution and eserine, to give a concentration between 1 in 2000 and 1 in 10,000. With greater amounts of tissue double the volume of the solution was used. The tissue was either divided with scissors or ground with silica in this acid solution, and then 0.3 c.c. of distilled water were added for each c.c. to make it isotonic for frog's tissue. The mixture was boiled for 1-2 min. and kept in the refrigerator until assayed for acetylcholine on the rectus abdominis muscle of the frog. Before the assay the solution was neutralized in the cold with 0.5 or 1 c.c. respectively of  $N/3$  NaOH and made up with the solution used for the frog's rectus test, so that each c.c. corresponded to a given amount (2-10 mg.) of tissue. The use of  $N/3$  HCl has the advantage of giving a physiological NaCl solution when neutralized with  $N/3$  NaOH. When, in control experiments, acetylcholine was added to Locke solution and treated in the same manner no measurable loss occurred.

For the incubation, the tissue was divided with scissors or ground in a solution similar to that used for extraction, but containing no HCl or distilled water. The pH of this buffered solution was 7.3. It was incubated in a water bath at 37-39° C. with oxygen bubbling through it. After the incubation 0.3 c.c. of distilled water per c.c. solution and 0.5 or 1 c.c.  $N/3$  HCl, respectively, were added to the mixture, which was boiled for 1 or 2 min. and then treated in the same way as the control sample. In some experiments both samples were cut or ground in a solution containing no eserine and no HCl; one sample

was then extracted in the usual manner by adding HCl, H<sub>2</sub>O and boiling, and the other sample was incubated after the addition of eserine. The acetylcholine yield, from most of the tissues examined, was so high that a solution equivalent to only 0.5–5 mg. tissue per c.c., was necessary for the test on the rectus muscle of the frog. In this case, the amounts of potassium or choline present were too small to interfere with the assay. When the acetylcholine yield was less than 2 µg./g. the estimation was usually carried out on the arterial blood pressure of the cat in chloralose anaesthesia.

The addition of tissue extract to the frog's rectus greatly sensitizes the muscle to a subsequent dose of acetylcholine. The sensitization wears off after several minutes and with repeated doses of acetylcholine. The following precautions were taken in order to avoid errors from this source. The first addition of extract was used as a sensitizing dose for the muscle, and for obtaining a crude estimation of the concentration of acetylcholine present in it. The extracts were then tested in alternating rotation with an acetylcholine solution, the strength of which (1 in 20 millions to 1 in 50 millions) was kept constant. The intervals between two contractions were kept between 4 and 7 min. A concentration of extract was used, the effect of which was definitely smaller (or greater) than that of the acetylcholine solution tested before or after it. The concentration of the extract was then increased (or decreased) with each test by 10–20%, until the effect was definitely greater (or smaller) than that of the acetylcholine solution. By keeping this solution constant throughout an assay any change in sensitization of the muscle is noticed, and does not interfere with the quantitative estimation. The acetylcholine yield of the tissue is expressed in µg. acetylcholine chloride/g. fresh tissue.

## RESULTS

### *Synthesis of acetylcholine in sympathetic ganglia*

Brown & Feldberg [1936a] have shown that, in cats, the acetylcholine content per g. tissue of the right and left superior cervical ganglion does not differ by more than 15%. The difference becomes smaller when ganglia from several cats are extracted together (Table 1). There appears also to be no great difference in the acetylcholine content of the two superior cervical ganglia in sheep, the differences in the two experiments of Table 1 being 0 and 2% re-

TABLE 1. Acetylcholine content of superior cervical ganglia

Animal	Mg. of ganglionic tissue extracted		Acetylcholine in µg./g.		Difference in %
	Right gl.	Left gl.	Right gl.	Left gl.	
1 sheep	65.0	58.4	18.2	17.9	2
1 sheep	175.6	178.8	11.4	11.4	0
2 cats	24.8	27.6	26.7	25.9	3
3 cats	37.8	33.8	26.2	27.6	5
4 cats	46.4	46.4	23.7	29.4	3

spectively. The variations from animal to animal are greater. In sheep, in which the acetylcholine concentration of the ganglia was found to be lower than in cats, the acetylcholine equivalent/g. fresh tissue varied in seven sheep from 8.3 to 18.2  $\mu\text{g./g.}$  (average 13.0  $\mu\text{g./g.}$ ). The corresponding figures from thirty-five cats were 23.5 and 36.8  $\mu\text{g./g.}$  (average 28.0  $\mu\text{g./g.}$ ).

*Incubation of ground or chopped ganglia.* Incubation of an excised ganglion brings about an increase in the amount of acetylcholine extractable from it. The amount yielded, however, depended upon whether the ganglia, before incubation, were divided with scissors for 1 or 2 min. or finely ground in a mortar with silica. In the latter condition synthesis of acetylcholine was small and did not occur regularly (Table 2); in the former it was pronounced, sometimes doubling, or even trebling, the acetylcholine yield during a period of

TABLE 2. Effect of 2 hr. incubation at 38–39° C. on the acetylcholine yield of ground up sympathetic ganglia

Animal	Acetylcholine in $\mu\text{g./g.}$		Difference in %	Treatment of ganglia
	Control gl.	Incubated gl.		
1 cat	4.0	3.6	-10	Eserine added after grinding
2 cats	32.9	27.9	-15	Ground in eserine
1 sheep	13.3	16.0	+20	Ground in eserine
1 sheep	2.9	3.5	+21	Eserine added after chopping the tissue with scissors for 5 min.

TABLE 3. Effect of incubation at 38–39° C. on acetylcholine yield of sympathetic ganglia chopped with scissors

Animal	Acetylcholine in $\mu\text{g./g.}$		In- crease in %	Period in min. of		Plasma present	Notes
	Control gl.	Incu- bated gl.		Chop- ping	Incu- bation		
1 sheep	8.3	20.0	141	2	130	0% horse pl.	Both sides chopped in saline without eserine
1 sheep	7.8	17.8	128	2	110	—	
1 sheep	11.1	30.3	173	3	120	—	
1 cat	4.3	11.4	165	2	160	15% cat's pl.	
1 cat	13.5	26.0	93	1	130	12% cat's pl.	
1 cat	6.7	14.3	113	2	120	7% horse pl.	Control gl. chopped in HCl; incubated gl. chopped in eserinated saline solution
1 sheep	18.9	42.5	130	2	120	—	
1 sheep	13.9	27.1	95	2	120	8% horse pl.	
1 sheep	8.3	27.0	218	2	120	10% horse pl.	
1 cat	27.0	52.5	94	1	130	—	
1 cat	26.3	52.5	165	1	90	—	
3 cats	28.6	47.1	65	2	110	—	
3 cats	27.1	54.3	115	2	120	—	
2 cats	36.8	67.4	79	2	120	6% cat's pl.	
4 cats	23.5	49.9	79	2	120	—	

incubation of about 2 hr (Table 3). The increase was obtained when incubation was started from a low level of acetylcholine in the ganglionic tissue or from its normal high level. To obtain a low level the ganglia were cut into small pieces or ground in saline solution containing no eserine, the acetylcholine released during the cutting up or grinding being destroyed by the cholin-

esterase. The control ganglia were then extracted with HCl and eserine was added to the flask containing the ganglia for incubation. This procedure accounts for the relatively low acetylcholine values of the controls in the first and last experiment of Table 2 and in the six first experiments of Table 3. In the other experiments the control ganglia were divided with scissors or ground in eserinated HCl, and the ganglia used for incubation in buffered eserinated saline solution.

The addition of plasma in a concentration of 7-15% to the medium did not appreciably influence the result, although the average increase without plasma was 106, with plasma 134%.

The difference in the results with finely ground ganglia and ganglia cut into small pieces suggests that the property of synthesizing acetylcholine is dependent upon the integrity of some structural part of the tissue, and that mechanical destruction, if carried out too extensively, leads to a loss of this property. That this occurs when a ganglion is divided too finely with scissors is shown by the last experiment of Table 2 in which the ganglion was cut up for a period of 5 min.

Synthesis of acetylcholine apparently occurs only when the acetylcholine store of the tissue is diminished, i.e. after acetylcholine has been released, the tissue being unable to build up an acetylcholine concentration higher or much higher than the normal value. This conclusion is based upon experiments in which, after incubation, the content of the flask was centrifuged, and the assay of acetylcholine carried out separately with the particulate matter and the supernatant fluid, a procedure adopted in four of the experiments recorded in Table 3. The results are tabulated separately in Table 4. Only once was there a yield of acetylcholine from the particulate matter higher (23%) than that of the control ganglion; in the other three experiments the ratio was reversed.

TABLE 4. Distribution of acetylcholine between particulate matter and fluid of incubated ganglion

Control gl. (a)	Acetylcholine content in $\mu\text{g./g.}$ in Incubated gl.		(b) as percentage of (a)
	Particles (b)	Fluid (c)	
28.6	15.7	31.4	55
23.5	22.2	17.8	94
27.1	33.3	25.0	123
13.9	12.9	14.2	93

*Cutting ganglia into small pieces.* If we assume that the synthesis occurs to replace acetylcholine, which has been released or diffused out of the particulate matter, the increase found after incubation must have been greatly dependent upon the acetylcholine loss from the tissue, and cannot give a true picture of the rate of synthesis of acetylcholine in ganglionic tissue. According to this



view, a stimulus leading to release of acetylcholine should be followed by synthesis or replacement. Chopping the ganglia with scissors ought to be a powerful stimulus of this kind and, in fact, leads, without subsequent incubation, to an increase in the acetylcholine yield of the ganglion. The results are shown in Table 5. The ganglia of one side had been divided with scissors in

TABLE 5. Acetylcholine yield of superior cervical ganglia chopped with scissors in HCl (a) and in eserinizd saline solution (b)

Animal	Acetylcholine in $\mu\text{g./g.}$ of				Increase of (b) in %
	(a)	(b)			
		Total	Particle	Fluid	
4 cats	25.0	40.2	13.3	26.9	61
4 cats	31.3	33.5	14.3	19.2	7
1 sheep	10.9	14.1	4.6	9.5	29

buffered eserinizd saline solution for 2 min., the mixture was at once centrifuged, and the particulate matter and the supernatant fluid extracted separately with HCl. The control ganglia had been chopped in HCl. In all three experiments the ganglia chopped in eserinizd saline solution had yielded a greater amount of acetylcholine than the ganglia chopped in HCl, the increase varying between 7 and 61%. In two of these experiments the amounts of acetylcholine brought into the solution by chopping the ganglia amounted to practically the total acetylcholine content originally present in them, indicating that the amount found in the particulate matter corresponded to the amount synthesized during the procedure of chopping. In the other experiment, in which there was a total increase of only 7%, less than two-thirds of the acetylcholine originally present in the ganglion had been released. The acetylcholine found in the particulate matter, in all three experiments, corresponded to about half the original amount. This value is lower than that obtained in the experiments of Table 4 after incubation. It appears therefore that the greater total yield on incubation results mainly from the fact that the acetylcholine is built up in the particulate matter during this period.

*Synthesis in ganglia during degeneration of preganglionic fibres.* We can confirm previous observations [Brown & Feldberg, 1936a; MacIntosh, 1941] on the disappearance of acetylcholine from the superior cervical ganglion of the cat following section of the cervical sympathetic trunk. Table 6 gives the actual values obtained, which, in Fig. 1, are expressed as percentages of the normal average acetylcholine content of the ganglion ( $28\mu\text{g./g.}$ ). The crosses in the figure are the results obtained by MacIntosh. The acetylcholine content of the ganglia 4-7 days after section of the sympathetic trunk was too low to be assayed on the frog's rectus muscle with the small amounts of tissue at our disposal. It was determined on the arterial blood pressure of the cat and some of the depressor action, which was atropine sensitive, may have been due to

TABLE 6. Content and synthesis of acetylcholine in superior cervical ganglia during degeneration of preganglionic nerve

No. of cats	Days after cutting cerv. symp.	Acetylcholine in $\mu\text{g./g.}$ of		Increase in %
		Control gl.	Incubated gl.	
1	1	16.7	47.2	+ 185
2	1	25.6	49.8	+ 95
2	2	8.8	7.1	- 19
3	2	5.3	8.3	+ 57
3	3	3.2	3.2	0
2	4	About 1.4	About 1.4	0
2	6	About 1	Little less than 1	0
2	7	About 1.5	About 1.3	- 13

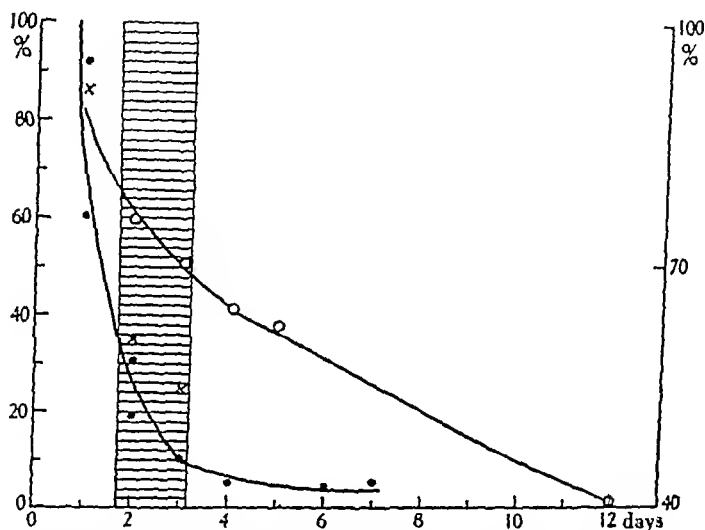


Fig. 1. Effect of preganglionic denervation on acetylcholine (●) and cholinesterase (○) content of the superior cervical ganglion of cats. Ordinates: percentages of normal acetylcholine content (on the left) and of normal cholinesterase content (on the right) of the ganglion. Abcissae: time in days after cutting the cervical sympathetic nerve. Shaded area: approximate period of impairment and loss of synaptic transmission with intact nervous conduction. The cholinesterase values are from Couteaux & Nachmansohn [1940]. (For details see text.)

the presence in the extracts of choline, which does not diminish in the ganglion after preganglionic nerve degeneration [Brown & Feldberg, 1936b]. For comparison, the decrease in cholinesterase content in the ganglion after preganglionic denervation is given in Fig. 1. Brücke [1937] had first observed this effect, but the values given are from Couteaux & Nachmansohn [1940], according to whom the cholinesterase decreases to 40% of its original value, and then remains constant. Even if the fact is taken into account that the cholinesterase does not disappear completely, its concentration decreases more gradually than that of acetylcholine, as can be seen from a comparison of the two curves.

After degeneration of the preganglionic fibres, the superior cervical ganglion loses its property of synthesizing acetylcholine which, therefore, is associated, not with the ganglion cells, but with the preganglionic nerve endings in the ganglion. The time when this property is lost coincides with the time when transmission of nervous impulses across the ganglionic synapses becomes impaired or fails. According to Coppée & Bacq [1938] synaptic transmission is impaired 40–50 hr. and abolished 45–72 hr. after cutting the preganglionic nerves. According to MacIntosh [1938] 'difficulties in transmission start after 48 hr. and it is completely or nearly completely abolished after 72 hr.' although at this time the nerve fibres still conduct impulses. In Fig. 1 the shaded area approximately represents this period of impairment and loss of synaptic transmission with intact nervous conduction. During this period, the ability of the ganglion to synthesize acetylcholine was found to become wanting. Synthesis was well pronounced 24 hr. after nerve section; after 48 hr. it was absent in one experiment (upper point on the curve), and diminished in the other (lower point). The latter experiment was carried out with the ganglia of three cats, and the diminution in synthesis may have taken place in all three ganglia, or synthesis may have been absent in one or two ganglia and normal or diminished in the remainder. The ganglia examined 3 days or later after section of the cervical sympathetic nerve no longer synthesized acetylcholine.

### *Synthesis of acetylcholine in cholinergic nerves*

*Cervical sympathetic nerve.* We can confirm MacIntosh's observation [1941] of a high acetylcholine concentration in the cervical sympathetic trunk of cats, and the same is true for the cervical sympathetic of sheep. It varied in seventeen cats between 21.5 and 40.0  $\mu\text{g./g.}$  (average 31.2  $\mu\text{g./g.}$ ) and in eight sheep between 11.9 and 22.7  $\mu\text{g./g.}$  (average 18.0  $\mu\text{g./g.}$ ). There is no strict parallelism between the values obtained for the ganglia and their preganglionic nerves, although in more than a third of the experiments the differences were less than 20%. Table 7 shows that the yield from the nerves was higher in more than half of the experiments; in a few instances the ratio was reversed.

TABLE 7. Acetylcholine content of superior cervical ganglion and its preganglionic nerve

Animal	Acetylcholine content in $\mu\text{g./g. of}$		Animal	Acetylcholine content in $\mu\text{g./g. of}$	
	Ganglion	Nerve		Ganglion	Nerve
Sheep	17.9	16.0	4 cats	25.0	33.3
Sheep	18.9	22.2	4 cats	31.3	31.3
Sheep	13.5	13.3	3 cats	28.6	29.4
Sheep	10.0	21.7	3 cats	27.1	40.0
Sheep	8.3	11.9	3 cats	27.6	21.5
Sheep	10.9	15.4			

As with ganglia, synthesis was small or absent when the nerves were ground with silica and incubated. The results obtained with nerves divided with

TABLE 8. Effect of incubation at 38–39° C. on acetylcholine yield of cervical sympathetic chopped with scissors

Acetylcholine in $\mu\text{g./g.}$ of		In- crease in %	Period in min. of		Plasma present	Notes
Control nerve	Incu- bated nerve		Chop- ping	Incu- bation		
Sheep	3.3	5.7	73	1½	140	Both samples chopped in saline without ese- rine
Sheep	3.3	7.1	115	1½	140	
Sheep	8.3	11.4	37	1	90	
Sheep	5.4	9.5	76	1	120	
4 cats	33.3	75.8	128	1	140	
3 cats	29.4	50.0	71	1	105	12% cat pl.

scissors and incubated for 90–140 min. in buffered eserinated saline solution are given in Table 8. The sheep's nerves were divided in saline solution containing no eserine; the control sample was then extracted with HCl and the other sample incubated after the addition of eserine. This procedure accounts for the low initial values of acetylcholine. In the experiments on cats the nerves used as controls were divided in HCl, those to be incubated were divided in eserinated buffered saline solution. The increase of acetylcholine after incubation amounted to between 37 and 128% (average 100%), a value slightly lower than that obtained for the ganglia. The addition of small amounts of eserinated plasma or serum to the medium used for incubation did not appear to have a definite effect on the acetylcholine yield. As in the experiments on ganglia, the increase of acetylcholine observed does not represent a true picture of the rate of its synthesis in nervous tissue. Table 9 shows that chopping the nerves

TABLE 9. Acetylcholine yield of cervical sympathetic chopped with scissors in HCl (a) and in eserinated saline solution (b)

Animal	Acetylcholine in $\mu\text{g./g.}$ of		Increase of (b) in %	Time of chopping nerve in min.
	(a)	(b)		
1 sheep	15.4	22.2	44	1½
4 cats	31.3	42.9	37	1
3 cats	21.5	36.4	69	1

with scissors for 60–90 sec. in eserinated saline solution, and then extracting them at once with HCl leads to an increase of 37–69% (average 50%), i.e. about half the increase in the two incubation experiments, in which the nerves were chopped in the presence of eserine, may have been brought about during the process of chopping.

*Degenerating cervical sympathetic nerve.* The acetylcholine concentration in the cervical sympathetic decreases progressively in the first few days after section of the nerve, the greatest decrease occurring, as in ganglia, between the first and third day. The results are given in Fig. 2, the acetylcholine content being expressed as percentage of the normal acetylcholine content of 31.2  $\mu\text{g./g.}$

fresh nerve. The sympathetic nerves were from the cats from which the ganglia had been taken, the rate of disappearance of acetylcholine being the same in both tissues. A similar decrease during nervous degeneration has been observed in motor nerves [Lissák, Dempsey & Rosenblueth, 1939] and in the vagus [MacIntosh, 1941].

The cervical sympathetic trunk loses its property of synthesizing acetylcholine at about the same early stage of degeneration (or perhaps a little earlier) as the ganglion. Forty-eight hours after section, the nerves no longer synthesized acetylcholine. They were from cats in which the ganglia had also lost their ability to synthesize acetylcholine.

The nerves from the other experiment, in which synthesis by the ganglia had been observed at this stage of nerve degeneration, were not examined. All the sympathetic nerves examined later than 48 hr. after section no longer synthesized acetylcholine. The acetylcholine of these nerves was assayed on the arterial blood pressure of the cat. One experiment was carried out 24 hr. after nerve section. There was an increase of acetylcholine with incubation of only 12%. Further experiments are necessary to decide if synthesis disappears earlier in nerve fibres than at their endings.

*Vagus nerve.* Table 10 shows the synthesis of acetylcholine occurring in samples of the vagus nerve chopped with scissors and incubated in eserinizied saline solution. The weight of the tissue used for each sample was about 370 mg. in the sheep experiment and between 25 and 70 mg. in the cat experiments. The period of chopping was 2 min. for the sheep's nerves and 1 min. for the cat's nerves. The nerves in the first four experiments were chopped before the eserine or the HCl respectively was added to the buffered saline solution, which accounts for the low control values of acetylcholine. In the last three experiments the controls were chopped in eserinizied HCl and those used for synthesis in buffered, eserinizied saline solution. A proportion of the increased yield of acetylcholine in the last two incubation experiments must have occurred during the procedure of chopping. This is evident from the last experiment of the table. The increase of 40% was obtained by chopping the nerves in eserinizied saline solution without subsequent incubation.

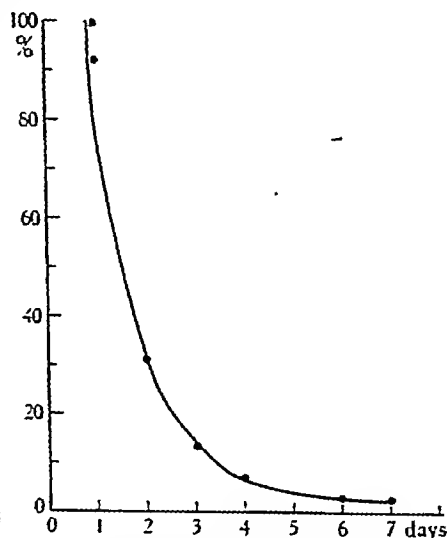


Fig. 2. Acetylcholine content in degenerating cervical sympathetic trunk of cats. Ordinates: percentages of normal acetylcholine content of the nerve. Abscissae: time in days after cutting the nerve. (For details see text.)

TABLE 10. Effect of incubation at 38–39° C. on acetylcholine yield of vagus nerve chopped with scissors

Animal	Acetylcholine in $\mu\text{g./g.}$ of		Increase in %	Incubation period in min.	Plasma present	Notes
	Control nerve	Incubated nerve				
Sheep	0.9	2.0	122	120	—	Both nerves chopped in saline without eserine
Cat	4.0	9.4	135	120	16% cat pl.	
Cat	3.8	7.3	90	160	12% horse pl.	
Cat	4.2	5.6	33	130	13% cat pl.	
Cat	9.4	13.0	39	120	—	
Cat	8.8	15.3	74	120	—	
Cat	7.8	10.9	40	0	12% cat pl.	

*Spinal nerves and nerve roots.* In one experiment two samples of cat's phrenic nerves were chopped with scissors for 1 min. in buffered saline solution without eserine. One sample was then extracted with HCl, the other incubated for 70 min. after adding eserine to it. The acetylcholine equivalent of the control was 6.2, of the incubated sample 8.2  $\mu\text{g./g.}$  tissue, an increase of 34%. In two experiments the lumbar motor roots were removed from a cat. Control samples were extracted with HCl, the others incubated in the usual way for 130 min. The acetylcholine yield of the controls was 22.2 and 20.8  $\mu\text{g./g.}$ , that of the incubated samples 42.5 and 42.7  $\mu\text{g./g.}$ , increases of 92 and 105% respectively. The corresponding sensory roots from the same cats were treated similarly, and the extracts assayed on the arterial blood pressure of the cat. The intravenous injection of extract equivalent to 20 mg. tissue scarcely gave any fall of blood pressure, if there was a difference the greater depressor action was with the controls. The depressor effect was less than that of 0.005  $\mu\text{g.}$  of acetylcholine, i.e. there was certainly not more than 0.25  $\mu\text{g./g.}$  acetylcholine in these roots, and there was no indication of it having been synthesized during the process of chopping and the subsequent incubation.

### DISCUSSION

Our initial conception that synthesis of acetylcholine in a sympathetic ganglion is a property not of the ganglion cells but of the preganglionic nerve endings in the ganglion, is borne out by the fact that the superior cervical ganglion of the cat loses this property after the cervical sympathetic has been cut. It is possible that the cells in parasympathetic ganglia, which, unlike most of the sympathetic ganglion cells, belong to a cholinergic neuron, would be found to exhibit this property and to do so independently of the integrity of their preganglionic nerve endings, and the same might be true for those sympathetic ganglion cells which give rise to postganglionic cholinergic axons. So far no experiments in this direction have been carried out. In the cat the superior cervical ganglion appears to have at the most only a few of such cells [Bacq, 1935]. The fact that loss of synthesis occurs simultaneously with impairment

and loss of synaptic transmission, but at a time when nervous conduction is intact, suggests the ability to synthesize acetylcholine as a necessary preliminary for normal, and particularly for sustained, transmission across ganglionic synapses. The disappearance of acetylcholine in sympathetic ganglia following section of their preganglionic nerves probably results also from the fact that the preganglionic nerve endings have lost their power to replace any acetylcholine when it has been released and destroyed. These conclusions are reinforced by the findings that cholinergic nerves synthesize acetylcholine in apparently the same manner as sympathetic ganglia, that both lose this property at the same early stage of nervous degeneration and that, in both, this stage is associated with a great drop in the acetylcholine content of the tissue. The loss of ability to synthesize acetylcholine, preceding the loss of nervous conduction, is among the first functional changes occurring in degenerating cholinergic nerves.

If we assume synthesis of acetylcholine to occur throughout the whole course of the fibres of cholinergic nerves up to their final endings, the structural element most likely to be concerned with it would be the tissue of which the axon and its endings are built up. MacIntosh [1941] has pointed out that the presence of acetylcholine in the fibres in a concentration of about the same order as that found in the ganglion suggests a manifold higher concentration at the preganglionic endings than along the course of the nerve fibres. We do not know if this increase in acetylcholine is brought about by a greater ability of the tissue at the endings to synthesize acetylcholine or to an accumulation, at the endings, of the tissue of which the axons are composed. Whenever the acetylcholine is released from this tissue in such a way as to be destroyed or washed away, if eserine is present, it is at once restored by synthesis. Similarly, synthesis of sympathin or adrenaline, the transmitter of adrenergic nerves, might perhaps occur after its release, along the whole course of the nerve up to its final endings. No function can yet be postulated for the release of the transmitter substances along the course of a nerve during the passage of an impulse. It has been demonstrated, however, for different cholinergic and adrenergic nerves [for literature see Lissák, 1939]. There is, as yet, no evidence that the release of acetylcholine, with subsequent synthesis, is necessary for the passage of an impulse in cholinergic nerves, a possibility envisaged by von Muralt [1937]. Otherwise the loss of synthesis should not precede that of conduction in a degenerating nerve. At the nerve endings the released acetylcholine finds a sensitive effector structure upon which to act, and, since it is restored immediately, synaptic transmission is permitted at the high frequency at which nerve impulses may follow each other for a long period of stimulation.

It would be misleading to deduce, from the amount of acetylcholine synthesized in our experiments during a given period, the rate of synthesis as it occurs in the body after a nerve impulse arrives at the nerve ending. The experi-

ments in which ganglia or nerves were cut into small pieces for a minute or two without subsequent incubation give some idea of the speed of synthesis, in response to the release caused by the stimulus of injury, under the conditions of the experiment. An even more striking instance of the rate of synthesis is given by the observation of von Muralto on the increased acetylcholine yield of nerves 'shot into liquid air', during stimulation. Synthesis or replacement of acetylcholine when released by nervous impulses may well occur within the refractory period of the nerve. Brown & Feldberg [1936*b*] have discussed such a possibility for synthesis of acetylcholine at preganglionic endings in sympathetic ganglia, when the acetylcholine has been released by preganglionic nerve stimulation.

Our experiments suggest that synthesis of acetylcholine is dependent upon some structural part of the tissue, since grinding the ganglia or nerves with silica greatly reduced or destroyed this property. The conditions for synthesis to take place, however, may vary in different tissues. For instance, brain tissue does not lose this property on grinding with silica. Nevertheless, in this tissue also, synthesis may be associated with the presence of particles such as cell debris or granules. According to Mann *et al.* [1939] spinning down and removing the particles of a suspension of minced brain leaves a supernatant fluid devoid of the property of synthesizing acetylcholine. In addition, they state that synthesis only occurs in a minced brain suspension when it is using oxygen, and that it is no longer obtained after the suspension has been frozen. Stedman & Stedman, on the other hand, observed synthesis in chloroform and ether suspensions of brain tissue under conditions which are incompatible with cellular activity.

#### SUMMARY

1. Sympathetic ganglia (superior cervical ganglion) and cholinergic nerves (cervical sympathetic, vagus, phrenic nerve and motor roots) divided with scissors into small pieces and incubated for 1-2 hr. in buffered saline solution, containing eserine, synthesize acetylcholine. This property appears to be dependent upon the intactness of some structural part of the tissue, since it becomes lost or greatly reduced when the mechanical destruction is carried too far, as by grinding the tissue with silica. No synthesis of acetylcholine has been observed with sensory roots.

2. Chopping sympathetic ganglia or cholinergic nerves with scissors for 1 or 2 min. leads, without subsequent incubation, to some synthesis of acetylcholine. Synthesis occurs apparently only in order to replace released acetylcholine and to restore its original concentration in the tissues. The procedure of chopping the tissue may release, and bring into solution, amounts of acetylcholine corresponding to those originally present in the tissue, but about half of this amount is resynthesized in the particulate matter of the suspension during the 1 or 2 min. of chopping.



3. When the cervical sympathetic trunk is cut in a preliminary operation, the distal part of the nerve and the superior cervical ganglion lose their property of synthesizing acetylcholine at an early stage of nervous degeneration (after about 48 hr.). The loss of synthesis occurs with the great drop in the acetylcholine content of the tissue and must be considered responsible for it. The loss precedes the loss of nervous conduction. In the ganglion it coincides with the time when synaptic transmission becomes impaired and lost. It is concluded that synthesis of acetylcholine in sympathetic ganglia is a property of the preganglionic endings and a necessary preliminary for normal and particularly sustained synaptic transmission.

I should like to make grateful acknowledgement to Sir Joseph Barcroft for supplying me with the nervous tissue of sheep.

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## THE ORIGIN OF THE INFLATION AND THE DEFLATION PULMONARY REFLEXES

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A paper has appeared recently by Partridge [1939] in which that author suggests that the acceleration of the breathing occurring on stimulation of the vagus peripheral to a spot cooled below  $8^{\circ}\text{C}$ . is due to the excitation of nerve fibres arising in the heart and not of fibres specifically related to the control of respiration, as we suppose, originating in the lungs.

In a former paper [Hammouda & Wilson, 1935] it was shown that excitation of a branch of the pulmonary vagus, lying on one of the larger bronchi at a distance of about 3 cm. from the hilum of the lung, caused the normal inhibition of respiration and, if the cervical vagus were cooled to  $6^{\circ}\text{C}$ ., the characteristic well-marked augmentation of the respiratory rhythm. These effects are ascribed by Partridge [1939] to the presence of looping cardiac afferent fibres in the pulmonary vagus.

In our view both the inhibition and acceleration of the breathing resulting from faradization of the vagus are due to the excitation of the same fibres which convey from the lungs to the centre the impulses arising from the physiological stimulus of expansion and collapse of the lungs, respectively. Partridge does not discuss the normal reflex acceleration of the breathing accompanying deflation of the lungs; she does not in fact refer to this reflex in any part of her paper. If Partridge's view were correct it would have to be assumed that this reflex is related to some indirect influence of the decrease in the lung volume on nerve endings in some part of the cardio-aortic area. This assumption, however, seems improbable in view of the fact that the reflex occurs equally well whether the collapse of the lung is brought about by increased external pressure or by negative intra-pulmonary pressure, in which two conditions the one effect on the circulation would be the reverse of the other.

It has also been suggested by other observers [Hess, 1930; Gesell, Steffensen & Brookhart, 1937] that the respiratory reflexes following inflation or deflation of the lungs may be, in part at any rate, of extra-pulmonary origin from nerve endings in the thoracic wall or diaphragm.

We hope to be able to show in the present communication that the deflation reflex is quite unaffected: (a) by section of all the cardiac branches of the vagus, (b) by absence of all afferent impulses reaching the centre by way of the spinal cord, and (c) by circulatory changes in the lungs. Further, it will be shown that while none of the cardiac branches excited below a cooled spot evoke the characteristic acceleration of respiration, the stimulation of the pulmonary branches all give this reflex response without any significant effects on the heart or circulation.

#### I. THE INFLATION AND DEFLATION REFLEXES BEFORE AND AFTER SECTION OF THE CARDIAC BRANCHES OF THE VAGUS

Dogs weighing from 4 to 11 kg. were employed in all the experiments recorded in the present paper.

*Method.* In the observations described in this section all the influences from the heart, aortic area and the carotid sinuses were excluded. Fig. 1 illustrates a typical result. After section of the right cervical vagus the dog was placed in the respiration chamber and the reflex effects of expansion and collapse of the lungs recorded (Fig. 1 A, B). The animal was then removed from the chamber, all the cardiac branches of the left vagus cut through an opening in the left 2nd and 3rd intercostal spaces, about 5 cm. of the 3rd rib being resected. The carotid sinuses on both sides were removed. To verify the fact that the cardiac and aortic vagal connexions conveying either afferent or efferent impulses had been completely destroyed, the cervical vagus was faradized and the third part of the aortic arch temporarily compressed. The heart frequency was not changed in either case.

The opening in the thorax was closed and the animal returned to the chamber. The reflex effects of inflation and deflation of the lungs were then recorded, first with the cervical vagus at the normal temperature and again after cooling to below 8° C.

It will be seen from Fig. 1, on comparing graph A from the intact animal (except for section of the right vagus) with graph C, in which all the cardiac nerves on the left side have been cut and the carotid sinuses extirpated, that the reflex effects of volume changes in the lungs are identical in the two conditions.

Graphs B, D and E show records of the reflexes when the conduction in the cervical vagus had been partially blocked by cooling the nerve to 4° C. Graph B was obtained from the normal dog while graphs D and E were taken after the exclusion of the cardiac vagal branches and the carotid sinuses. It will be seen that the reflex effects of inflation and deflation of the lungs, including the reversal of the inflation reflex, are obtained not only in the normal animal (graph B) but also in the dog in which no influences except those arising from the lungs are present (graphs D and E).

Section of the pulmonary vagus abolished all the reflex reactions following inflation and deflation.

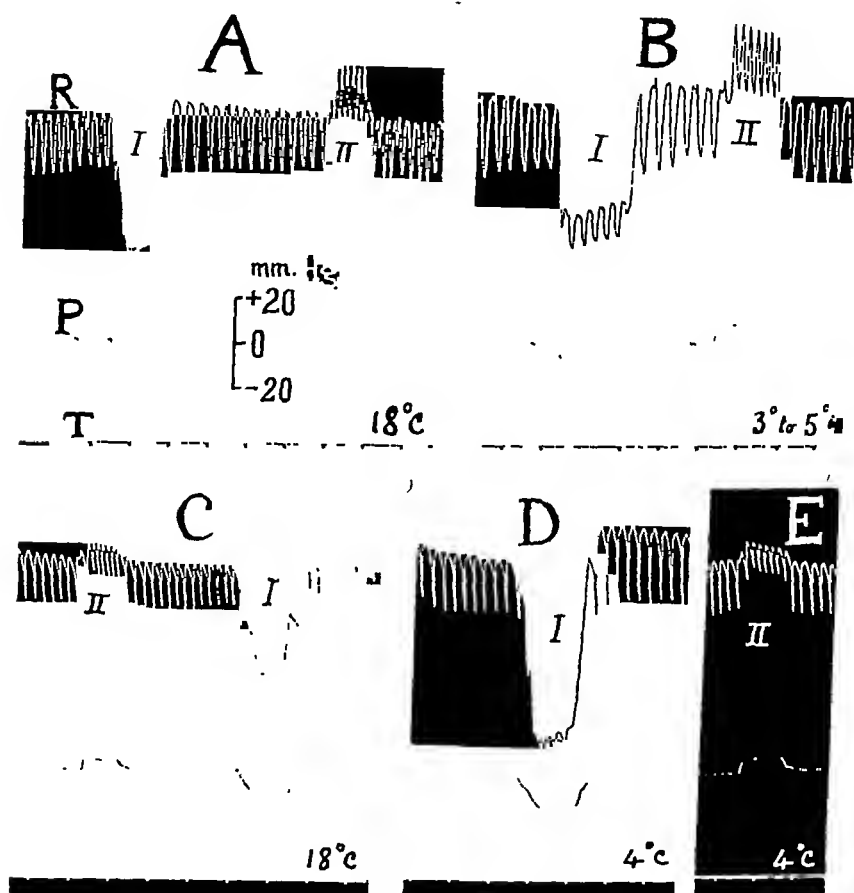


Fig. 1. Respiratory reflexes after section of vagal cardiac nerves and extirpation of carotid sinuses. Dog, male, 4.5 kg., morphia (0.4 c.c. of 1% subcutaneously) and medinal (10 g. intravenous) anaesthesia. The respiration chamber used throughout. Right cervical vagus cut in neck. Graphs A and B taken before while C, D and E taken after section of the left cardiac vagal branches and extirpation of both carotid sinuses. A, inflation caused arrest of respiration while deflation produced acceleration from 21 to 42 per min. B (left cervical vagus cooled to 4° C.), inflation and deflation caused acceleration of breathing, from a frequency of 18 in both cases, to 21 and 33 per min., respectively. C, inflation caused arrest while deflation produced acceleration from 28 to 45 per min. D (left cervical vagus cooled to 4° C.), inflation caused acceleration from 21 to 30 per min. E (left vagus cooled to 4° C.), the deflation caused acceleration from 26 to 30 per min. R=spontaneous respiration (bellows record). P=pressure in respiration chamber in mm. Hg. T=time record in 10 sec. I=inflation of lungs. II=deflation.

## II. RESPIRATORY REFLEXES AFTER SECTION OF THE SPINAL CORD ABOVE THE THORACIC REGION

Gesell *et al.* [1937] believe that the reflex effects on respiration of collapse of the lungs may arise from the excitation of nerve endings in the parietal pleura or in the structures of the thoracic wall. Similarly, Hess [1930] described reflex actions resembling those accompanying expansion and collapse of the lungs as being set up by artificial movements of the thoracic wall of an animal with open chest, when the volume of the lungs was unchanged. Hess considers that this observation supports his view that respiration is in part controlled by reflex impulses arising in these extra-pulmonary structures.

In the experiments described below, in which the reflex effects on respiration were compared before and after section of the spinal cord, we have been unable to find any evidence that either the reflex inhibition or acceleration of breathing, normally induced by changes in the lung volume, can be produced except from nerve endings lying within the lungs.

*Method.* Dogs were anaesthetized with chloralose, no morphine being used. The animal was placed in the respiration chamber and a record was taken, with the bellows recorder, of the reactions to expansion and collapse of the lungs, employing diminished and increased extra-thoracic pressures, respectively.

After removal from the chamber the spinal cord was exposed by excision of the arch of the last cervical vertebra. The dura was opened and a stout silk ligature passed round the cord within the dural space. The ligature was then firmly tied, severing the cord. A ligature was employed in preference to cutting the cord with a knife in order to avoid undue haemorrhage. The wound was lightly packed with cotton-wool and partially closed with stitches. The animal was then returned to the chamber and records taken of the effects of inflation and deflation of the lungs.

*Results.* Following the low cervical transection of the cord, the diaphragmatic movements continued normally, but all the thoracic respiratory movements ceased. It is remarkable how little the exclusion of the thoracic respiration had on either depth or frequency of breathing. This may very largely be accounted for by the fact that in the chloralosed dog, except in asphyxial conditions or after vagotomy, respiration is usually mainly diaphragmatic.

Fig. 2 shows the results of the procedure. Graph I was taken before and graph II taken about 30 min. after transection of the spinal cord at the level of the last cervical vertebra.

Graphs III and IV were obtained from another dog, the former showing the inflation and deflation reflexes in the spinal animal and graph IV the reversal of the inflation reflex during cooling of the vagus to about 3° C.

It will be seen from graphs I and II that there is no significant change in the frequency or depth of breathing or in the character of the responses to inflation

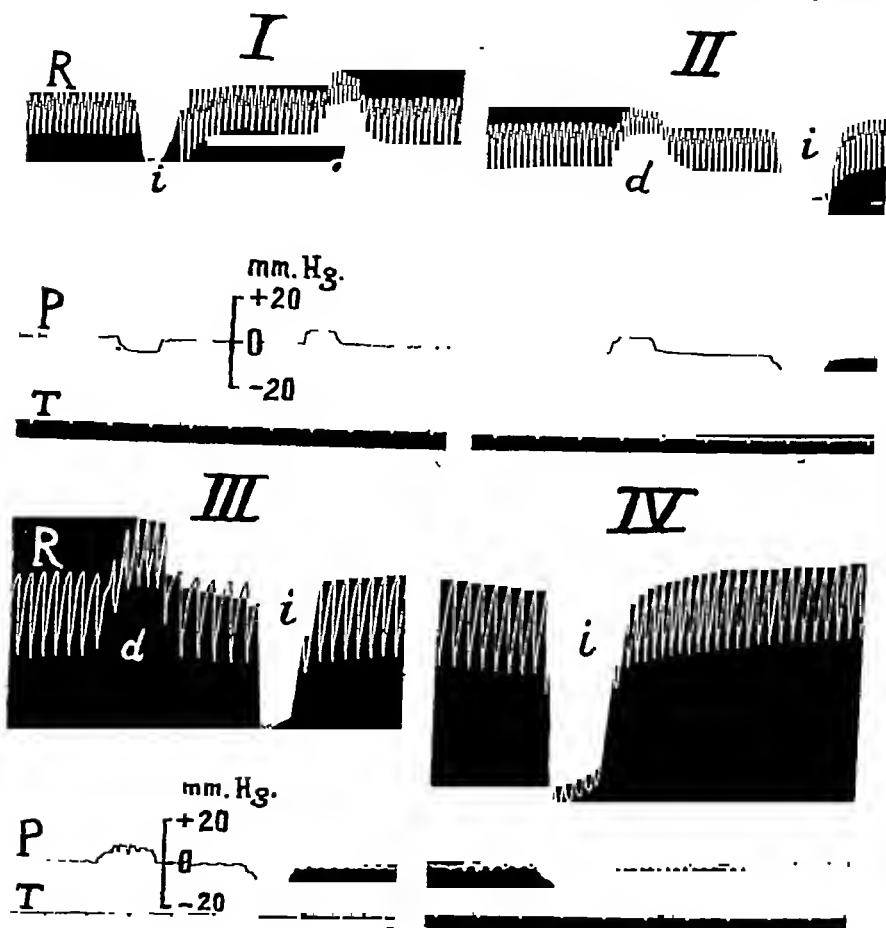


Fig. 2. Inflation and deflation reflexes in the spinal animal. Exp. 1 (graphs I and II): Dog, female, 5.5 kg., no morphia; chloralose anaesthesia (0.375 g.). Respiration chamber used. I, inflation and deflation in intact animal. II, same reflexes after the spinal cord had been tied at the level of the last cervical vertebra. Exp. 2 (graphs III and IV): Dog, female, 4.5 kg., no morphia; chloralose anaesthesia (0.315 g.). The left cervical vagus was cut and the spinal cord tied at the level of the last cervical segment. Respiration chamber used. III, deflation and inflation reflexes in spinal dog. IV (right cervical vagus cooled to about 3° C.), reversal of inflation reflex. *R*=bellows record of respiration. *P*=pressure in respiration chamber. *T*=time in 10 sec. *i*=inflation of lungs by negative pressure in chamber. *d*=deflation by positive pressure.

or deflation of the lungs resulting from complete exclusion of all afferent impulses carried by spinal nerves below the level of the cervical region of the cord.

From other experiments we have found that the inflation and deflation reflexes are unaffected by section of the phrenic nerves on both sides, indicating that the afferent impulses, arising from the diaphragm and adjacent structures and ascending in the phrenic nerves, are not concerned in the reflex response to volume changes in the lungs.

The conclusion to be drawn from §§ I and II is, therefore, that the deflation and inflation reflexes arise solely from the excitation of vagal nerve endings within the lungs.

### III. THE REFLEX EFFECTS OF PULMONARY EXPANSION AND COLLAPSE IN THE COMPLETELY ANAEMIC LUNG

As already pointed out in the introduction of this paper, the fact that the reflex effect of deflation of the lungs occurs equally well whether the collapse

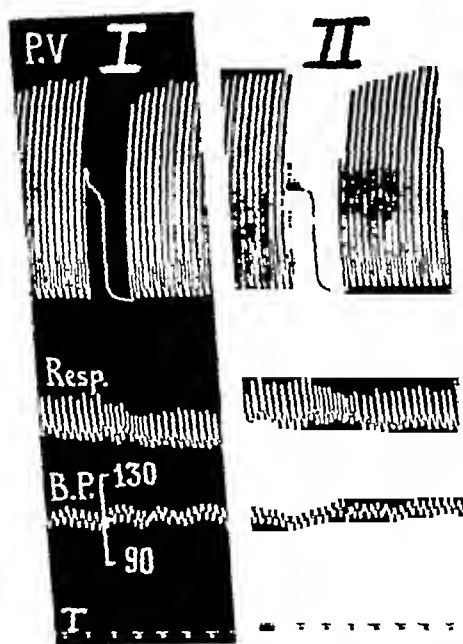


Fig. 3. The deflation reflex before and during complete occlusion of the left pulmonary artery. Dog, male, 9.0 kg., morphia (0.9 c.c. of 1% solution subcutaneously) and chloralose (0.68 g. intravenous) anaesthesia. Open chest; artificial respiration. Right cervical vagus cut. Cannulae in trachea and right bronchus. Graph I was taken before while graph II was taken during occlusion of the left pulmonary artery. Respiratory reflexes are identical in both cases. P.V.=artificial ventilation by pump. Resp.=spontaneous respiratory movements of chest wall. T=time in 10 sec. B.P.=blood pressure in mm. Hg. Further details in text.

is produced by an external positive pressure or by a negative intra-pulmonary pressure (suction)—procedures causing opposite effects on the pulmonary

circulation—appears to exclude the assumption that the reflex acceleration of breathing accompanying collapse may be related to a change in the circulatory conditions in the lung itself or to the indirect effect of a change in the rate of return of the blood to the heart.

The reflex effects of volume changes in the lungs were examined after the circulation through the innervated lung had been arrested by temporary occlusion of the pulmonary artery. .

*Method.* With the open thorax a ligature was passed under the left pulmonary artery and left loose. The respiration pump was connected by one branch of a T-piece to the tracheal cannula and by the other to a cannula tied into the right main bronchus. The right cervical vagus was cut, leaving the left intact. By this arrangement the left lung could be cut off from the pump by clamping the rubber connexion, while aeration of the blood continued by ventilation of the right denervated lung. When the connexion with the pump is closed, inflation or deflation of the left innervated lung could be effected through a side tube between the clamp and the tracheal cannula. The arrest of circulation of blood through the left lung was achieved by drawing on the ligature surrounding the left pulmonary artery and applying to the latter a stout bull-dog clamp.

This method thus allows of an examination of the reflexes without the interference by the pump rhythm and without great interference with the ventilation or the return of blood to the heart either with or without the cessation of circulation through the innervated lung.

*Results.* Fig. 3 shows a record of an observation made on a dog prepared as described above. Graph I was taken without and Graph II taken with occlusion of the left pulmonary artery.

It will be seen that the reflex effect of collapse of the lung is in no way influenced by the circulatory changes in the lung itself. The deflation reflex is in fact identical in the two cases.

#### IV. REFLEX EFFECTS OF EXCITATION OF CARDIAC OR PULMONARY BRANCHES OF THE VAGUS NERVE

Brodie & Russell [1900], confirming and extending Wooldridge's earlier observations [1883], faradized all the cardiac branches of the thoracic vagus as well as the pulmonary branches. In most cases they found a reflex slowing of the heart and a fall of blood pressure. They describe these effects as being most marked on stimulation of any of the pulmonary branches. One cardiac branch, the lowest on the right side, gave a very marked rise of blood pressure. Brodie & Russell do not mention, nor does an examination of any of their figures show, any reflex acceleration of the respiratory rhythm on stimulation of the cardiac vagal branches.



Partridge [1939], as the result of her observations on the excitation of the pulmonary branches of the vagus, believes that the fibres of the vagus that give rise to acceleration of respiration are in reality afferent fibres of cardiac origin which, after joining the vagus, travel peripherally a certain distance and then loop round to issue with the pulmonary branches on their way to the respiratory centre; the evidence being that those branches of the vagus, which when stimulated below a cooled area produced respiratory acceleration, showed with the oscillograph the presence of electrical variations synchronous with the heart beat. This author's statement implies that certain pulmonary branches of the vagus carry only inhibitory and not respiratory accelerator fibres. The experimental observations recorded below (Subsection b) do not confirm this view.

(a) *Reflex effects of excitation of the cardiac branches of the vagus*

The branches of the left intra-thoracic vagus arising between the level of the stellate ganglion and the root of the lung, just above the origin of the pulmonary branches, vary in different dogs but are usually about six in number. These include all the afferent and efferent cardiac branches of the left vagus. They were dissected, cut and the central ends faradized under normal temperature conditions and also during cooling of the left cervical vagus to about  $5^{\circ}\text{C}$ .

*Results.* Electrical stimulation of the central stumps of the cardiac vagal branches produced either no effect at all or only a reflex slowing of the heart and fall of blood pressure accompanied by a decrease in the frequency of breathing. A branch, arising from behind the aortic arch and doubtless the cardio-aortic 'depressor' nerve, gave a definite reflex inhibition of respiration (Fig. 4, I). Repetition of the stimulation while the cervical vagus was cooled to  $5^{\circ}\text{C}$ . was without effect (Fig. 4, II), indicating that the cardio-aortic branch did not contain respiratory accelerator fibres.\*

If it were the case that the afferent cardiac branches of the vagus contain fibres which when stimulated cause a reflex acceleration of the breathing, it would be expected that excitation of the cervical vagus below a cooled spot would produce a more pronounced increase in frequency of breathing than would excitation of the pulmonary vagus below the entry of these cardiac fibres. The results of an experiment to test this are shown in Fig. 5 in which the pulmonary and the cervical vagi, respectively, were faradized under identical conditions below a spot cooled to about  $4^{\circ}\text{C}$ .

\* In one case the stimulation of a cardiac vagal branch, issuing from the vagus at the level of the stellate ganglion, produced a remarkable rise of the blood pressure which was accompanied by an irregular acceleration of breathing. Although the phenomenon resembles that following stimulation of somatic sensory or pain nerve fibres, yet it is not identical since these reflex effects still occurred during cooling of the cervical vagus to about  $0^{\circ}\text{C}$ . [cf. Wilson & Hammouda, 1933].

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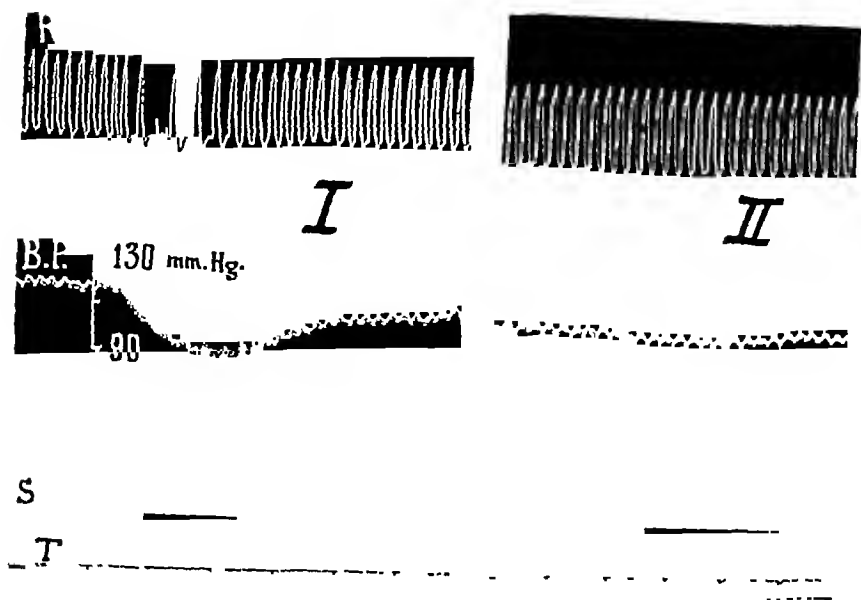


Fig. 4. Effects of stimulation of the cardio-aortic 'depressor' nerve before and during cooling of the cervical vagus. Dog, male, 7.3 kg., morphia-medinal anaesthesia. Open chest; artificial ventilation. Both pulmonary vagi cut and carotid sinuses extirpated. All cardiac branches of left vagus are cut and central ends prepared for stimulation. I, stimulation of the cardio-aortic depressor branch of the left vagus before, and II, during cooling of left cervical vagus to 5° C. R=spontaneous respiration (stethograph). B.P.=blood pressure in mm. Hg. S=signal. T=time in 10 sec.

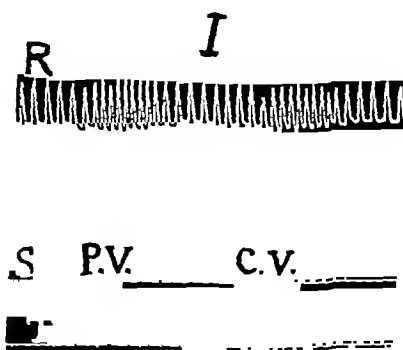


Fig. 5. Comparison of effect of stimulating the cervical vagus and the pulmonary vagus during cooling of the nerve in the neck. Dog, male, 5.5 kg., morphia-medinal anaesthesia. Atropine (1.0 mg. intravenous). Open chest; artificial ventilation. Both pulmonary vagi cut. Right cervical vagus cooled to 4° C. P.V.=faradio stimulation of central end of the right pulmonary vagus below the origin of the cardiac branches. C.V.=identical stimulation of the right cervical vagus above the origin of the cardiac branches (but below the cooled spot). R=respiratory movements of chest wall (stethograph). S=signal. T=time in 10 sec.

It will be seen that with an initial frequency of breathing of about 21 per min., stimulation of the cervical vagus below a cooled spot produced an acceleration of the breathing to about 36 per min., while an identical stimulation of the pulmonary vagus increased it to about 39 per min. This clearly shows that the cardiac branches, which are absent from the pulmonary vagus, added nothing to the respiratory accelerator fibres content of the cervical vagus.

(b) *Reflex effects of excitation of the pulmonary branches  
of the vagus*

To determine whether any of the branches of the pulmonary vagus carried only one type of fibre (respiratory inhibitory or accelerator) a number of observations has been carried out on the branches entering the hilum of the lung on both sides. The results have been consistent.

*Method.* Under morphine-chloralose anaesthesia, artificial ventilation was started and the 4th, 5th and 6th ribs of the right side were resected at the back so as to leave a window through which the posterior aspect of the hilum of the right lung was fully exposed. All the visible branches of the vagus at the hilum were carefully dissected, a thread passed under each and tied, leaving as long a section of the branch between the ligature and the main trunk of the vagus as possible. Each branch was then cut peripheral to the ligature. Faradization of the central ends was made successively at the normal temperature and subsequently during cooling of the right cervical vagus to 4° C. The left cervical vagus was cut.

It was found necessary to maintain artificial ventilation throughout the experiment, as, although the left side of the thorax remained intact, the mediastinum of the dog is not sufficiently rigid to allow of an efficient ventilation by the animal's unaided respiration. Stethograph and blood pressure records were also taken.

*Results.* Table 1 shows the results of an experiment in which the effects of stimulation of the nine pulmonary branches of the right vagus were recorded under normal temperature conditions and during cooling of the right cervical vagus to 4° C. Identical results were obtained by similar experiments on the left side.

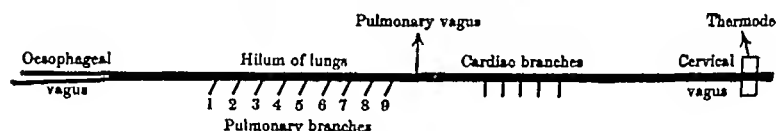
It will be seen that in those cases where there was a definite inhibition of respiration at normal temperature there was a well-marked acceleration of the rhythm when the inhibitory fibres were blocked. In only one case (branch 1 in Table 1) was there no inhibitory action but an insignificant acceleration with the cooled nerve.

TABLE 1. Effect of stimulation of pulmonary vagal branches with and without cooling of right cervical vagus

Dog, male, 7.0 kg., morphine-chloralose (0.50 g.) anaesthesia. Left cervical vagus cut; pulmonary branches of right vagus were dissected, cut and their central ends stimulated through a window in right thorax made by resecting the 4th, 5th and 6th ribs. Artificial ventilation.

Branch stimulated	Right cervical vagus at normal temperature			Right cervical vagus cooled to 3 or 4° C.		
	Resp. frequency before stimulation per min.	Resp. frequency during stimulation per min.	Blood pressure change mm. Hg	Resp. frequency before stimulation per min.	Resp. frequency during stimulation per min.	Blood pressure change mm. Hg
Pulmonary branch 1	29	29	-5	39	42	No change
" 2	29	0	-10	36	48	"
" 3	29	0	No change	36	45	"
" 4	29	0	-5	33	44	"
" 5	29	0	-5	36	45	-3
" 6	29	0	No change	35	45	No change
" 7	29	Slowing	"	39	39	"
" 8	29	0	-35 (bradycardia)	39	45	-10
" 9	29	Slowing	No change	36	36	No change

Diagram of the right vagus and its branches



## DISCUSSION

The experimental observations recorded in this paper are designed to show that the acceleration of the respiratory rhythm, produced reflexly either by collapse of the lungs or by direct faradization of the cervical vagus below a cooled spot, is due in the first case to the excitation of specific nerve endings in the lungs and in the second case to the electric stimulation of the identical nerve fibres which travel in the vagus on their way to the respiratory centre.

The first three sections are concerned with the deflation reflex: the results demonstrate beyond doubt that this reflex is solely related to the collapse of the lungs and is not of extra-pulmonary origin. It is shown that the reflex can neither be due to the excitation of nerve endings in the cardio-aortic area or in the thoracic walls, nor be due to circulatory changes produced directly or indirectly in the lungs or elsewhere.

In § IV the effects of excitation of the intra-thoracic branches of the vagus, arising from the heart and mediastinal tissues above the hilum of the lung and of the branches of the nerve entering the lung at the hilum, have been examined. The experiments were carried out with the purpose of investigating Partridge's view [1939] that the fibres of the vagus or its pulmonary branches

which cause an increase in the frequency of respiration when excited below a cooled spot are of cardiac origin.

We have been unable to confirm Partridge's statements: (a) that stimulation of cardiac branches of the vagus gives rise to any significant respiratory acceleration, (b) that pulmonary branches are found which, when excited, give inhibition of the breathing but do not give acceleration when the inhibitory fibres are blocked by cold, or (c) that branches which, when excited below a cooled spot, give accelerated respiration but do not give inhibition under normal temperature conditions. Our results indicate that all the branches of the vagus entering the lung at the hilum, stimulation of which produces a definite inhibition of the breathing, also contain the respiratory accelerator fibres.

Partridge [1939] bases her conclusion, namely, that the pulmonary branches which give rise to increased frequency of breathing, when excited below a cooled spot, are of cardiac origin, on the fact that all such branches show with the oscillograph well-marked electrical variations synchronous with the heart beat. It must be pointed out, however, that the presence of such electrical variations is no evidence that the fibres carrying such impulses arise in the heart or cardio-aortic area. Indeed, the oscillographic studies on the afferent impulses from striated muscle [Matthews, 1933], from the mesentery [Gammon & Bronk, 1935] and from the urinary bladder [Talaat, 1936] have shown that where nerve endings lie close to an artery, the nerve fibres arising in them will show changes of electrical potential synchronous with the systolic expansion of the vessel. This phenomenon may very well explain Partridge's findings since it is clear that intra-pulmonary nerve endings lying in close proximity to a branch of the pulmonary artery will show potential changes synchronous with the heart beat as is the case in muscle and other tissues.

In a previous paper on this subject [Hammouda & Wilson, 1932], in which the reflex acceleration of breathing accompanying the collapse of the lungs was described, the view was put forward that this reflex arises from the excitation of nerve endings in the lungs other than those causing reflex inhibition of the breathing with pulmonary expansion. Reasons were given for believing that these nerve endings are in a tonic state of excitation, and that the impulses arising in them travel in the vagus and exert an augmentor influence on that part of the respiratory centre which governs the frequency of discharge, the frequency being greater than would be the case in the absence of this tonic influence. The characteristic slowing of the breathing following double vagotomy would, therefore, be due to the absence of the augmentor influence arising from the deflation receptors of the lungs.

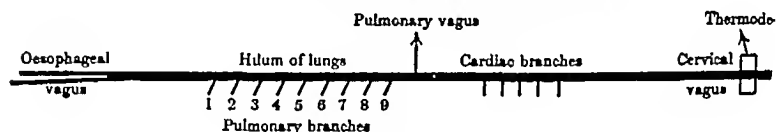
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That a tonic augmentor influence is carried to the respiratory centre by the vagus was shown by an interesting observation of Heymans & Heymans [1928]. These authors maintained the ventilation of one lung through a cannula

introduced into the bronchus, the same lung being denervated by cutting the cervical vagus. The other innervated lung was maintained in a collapsed position. They found that the respiratory centre was discharging at the normal frequency. On cutting the vagus of the deflated lung the characteristic slow vagotomy breathing was at once established. These authors believed that the impulses, the removal of which led to the slowing of the rhythm, arose from nerve endings in the cardio-aortic area. Anrep & Segall [1926] had previously shown that section of the vagus just above the hilum of the lung did not disturb the cardio-aortic innervation. They found, in fact, that apart from a few oesophageal branches all the vagal branches in the region of the hilum go to the lungs. Anrep & Samaan [1932], examining Heymans's results, were able to show that after complete section of all the branches of the vagus leading to the cardio-aortic area slowing of the breathing did not occur. The classical slow vagotomy breathing was, however, at once established on section of the pulmonary or cervical vagi. This demonstrates that the impulses reaching the centre, to the absence of which the change in the rhythm is due, are of pulmonary origin.

The view of Anrep & Samaan [1932] is confirmed by the observations recorded in the present communication. This conclusion also supports our view as to the cause of the deflation reflex and the pulmonary origin of the respiratory accelerator fibres present in the vagus.

A further proof of the pulmonary origin of these respiratory accelerator fibres has recently come to light in an investigation carried out by Bagoury & Samaan [1941] on the reflex respiratory phenomena appearing as the result of injection of ketone bodies into the pulmonary circulation. Ethyl acetoacetate, the most active drug of the series, produces temporary inhibition of respiration comparable to that caused by inflation of the lungs. If, however, the cervical vagi are cooled to about 6° C. a definite acceleration of the breathing is produced. It is reasonable to assume, therefore, that the vagal terminations of both types in the lungs are excited by this drug. Bagoury & Samaan [1941], by appropriate methods, have excluded the possibility of the effects following administration of ethyl acetoacetate being of central or of extra-pulmonary origin.

In conclusion the present paper gives further evidence that the deflation reflex arises solely from intra-pulmonary nerve endings, and that the respiratory accelerator fibres of the vagus are the path by which the impulses arising in these nerve endings reach the respiratory centre.

#### SUMMARY

Two questions, raised by the authors referred to in the introduction, have been the subject of investigation. The first question is to what extent, if any, the reflex effects of volume changes in the lungs are of extra-pulmonary

origin. The second is whether the respiratory accelerator fibres present in the vagus are of cardiac or entirely of pulmonary origin.

1. In regard to the first question it is shown that:

(a) Section of all the cardiac branches of the vagus and extirpation of the carotid sinuses do not affect the inflation and deflation reflexes or the reversal of the former with the selectively cooled vagus.

(b) These reflexes are equally unaffected in the absence of all afferent impulses from the thoracic walls and parietal pleura or by denervation of the diaphragmatic area.

(c) In the otherwise intact animal, the above reflexes are completely abolished by section of the pulmonary vagi.

(d) Circulatory changes in the lungs have no direct or indirect effect on the reflexes.

The conclusion is that the reflex changes in respiration accompanying inflation or deflation originate entirely in intra-pulmonary nerve endings.

2. In regard to the second question it is shown that:

(a) Stimulation of the central ends of the cardiac branches of the vagus gives rise to no acceleration of the breathing whether the cervical vagus is cooled or is at the normal temperature. Nor does the stimulation of the vagus (with selective cooling) above the entry of the cardiac branches have any greater effect in accelerating the rate of breathing than does excitation below the entry of these branches.

(b) The only significant effect of stimulation of the central ends of the pulmonary branches of the vagus at the hilum of the lung is, under normal temperature conditions, inhibition of respiration, but with cooling of the cervical vagus below 8° C. acceleration of the rhythm.

The conclusion is that both respiratory accelerator and inhibitory fibres of the vagus arise from nerve endings within the tissues of the lungs.

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## OESTROGENS AND ACETYLCHOLINE

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Reynolds [1939] reported that the administration of oestrogen to spayed rabbits increased the acetylcholine content of the uterus, and he attempted to explain in this way the vaso-dilator action of the oestrogens on the uterine blood vessels. In his experiments, extracts of the uteri of uninjected controls contained very little or no acetylcholine, on the average less than  $0.1 \mu\text{g./g.}$  of tissue, while extracts from the uteri of animals, injected 1 hr. previously with 100-400 i.u./kg. of oestrogens from the urine of pregnant mares (Amniotin, Squibb), gave values averaging about  $0.4 \mu\text{g./g.}$  Reynolds & Foster [1939] confirmed these results, and extended the observations to include oestradiol and its esters and triphenylethylene, but found no effect with diethylstilboestrol. Similar differences were later reported for the vaginal tissues [Reynolds & Foster, 1940c] and the nasal mucosa of both rabbits and cats [Reynolds & Foster, 1940b]. In spayed cats and rats, however, there was no increase in uterine acetylcholine when oestrogens were injected [Reynolds & Foster, 1940a].

These reports suggested an investigation into the mechanism by which oestrogenic stimulation might cause an accumulation of acetylcholine in the uterus. It appeared possible that this action of the oestrogens might be due to their inhibiting the normal destruction of acetylcholine in the uterine tissues. In the first place, therefore, the effect of oestradiol on the cholinesterase of the rabbit uterus was tested.

## OESTRADIOL AND CHOLINE ESTERASE ACTIVITY

Groups of Dutch-marked rabbits, ca. 1.5 kg. in weight, were spayed 40-50 days before use and were divided into sets of (a) uninjected controls and (b) injected animals receiving 1 mg. of free oestradiol in nut oil subcutaneously 1 hr. before killing. This is a dose of about 30,000 i.u., considerably in excess of Reynolds's doses, but it did not always produce visible vaso-dilatation. It should be noted, however, that only a small fraction of the total dose can have left the

site of injection within 1 hr. The cholinesterase activities of the uteri were measured by the manometric method, under the conditions described by Jones & Tod [1935]. Various procedures were tried at different times, all involving the setting up of ground, chopped or sliced uterine tissue in saline-bicarbonate buffer in Warburg vessels, and the measurement of  $\text{CO}_2$  liberated by the acetic acid formed through the hydrolysis of acetylcholine. Control experiments with samples of ileum were also set up in all but one test. Enzyme activities are expressed in terms of the volume of  $\text{CO}_2$  (c.mm.) liberated per min. per g. of moist tissue which had been pressed between layers of dry filter paper.

Pretreatment with oestradiol had no effect on the rate of splitting of acetylcholine under these conditions, with preparations of either uterus or gut, and the experiments gave no evidence that oestrogens so administered affected the action of cholinesterase. The average figures for c.mm.  $\text{CO}_2$ /min./g. of uterine tissue at  $38^\circ \text{C}$ . and 760 mm. were  $32.9 \pm 6.3$  ( $\sigma_n$ ) with fifteen control rabbits, and  $27.9 \pm 3.8$  and eighteen injected rabbits, the difference being 5.0, and its standard error 7.34. The corresponding figures for gut were 78.2 and 83.3.

Further tests for an action *in vitro* were then made by adding 2 mg. of oestradiol or diethylstilboestrol in 0.2 ml. propylene glycol to 4 ml. of a suspension of the ground or chopped uterus. Propylene glycol alone at this concentration had no effect on the rate of splitting of acetylcholine, nor had oestradiol, but a slight inhibition was observed in two experiments with diethylstilboestrol, amounting to 35 and 40%. Acetylcholine is normally destroyed so rapidly by the cholinesterase in the tissues that the oestrogens would need to produce a very considerable inhibition of the enzyme if an increase in uterine acetylcholine were to be explained on this basis.

#### ACETYLCHOLINE SYNTHESIS *IN VITRO*

As the tests reported above were negative, the effect of oestradiol on the synthesis of acetylcholine was studied. Several methods were tried, including a method found by Mann, Tennenbaum & Quastel [1939] to give good synthesis of acetylcholine in brain, namely incubation of the tissue in the presence of  $\text{O}_2$ , glucose, phosphate or bicarbonate buffer, eserine and excess KCl. Acetylcholine was liberated by acidifying to pH 4, and the activity of the extracts was assayed on the blood pressure of the eviscerated cat [Brown & Feldberg, 1936]. With this technique we also found clear evidence for acetylcholine synthesis by brain *brei*; but its rate was unaffected by propylene glycol, or by propylene glycol + oestradiol (Table 1). Under similar conditions, little acetylcholine was obtained from extracts of incubated uterine *brei*, and the yield was unaffected either by propylene glycol or oestradiol.

TABLE 1. Acetylcholine synthesis by tissue slices from spayed rabbits

Tissue	Weight in mg.	Volume of extract ml.	Acetyl- choline $\mu\text{g./g.}$	Treatment
Brain	125	3.0	24.0	None
	125	3.0	1.9	Acidified at once
	125	3.0	31.0	0.2 ml. propylene glycol added
	125	3.0	24.0	0.2 ml. propylene glycol + 2 mg. oestradiol added
Uterus	133	3.0	0.32	None
	135	3.0	0.31	0.2 ml. propylene glycol added.
	130	3.0	0.32	0.2 ml. propylene glycol + 2 mg. oestradiol added

Tissue slices incubated for 1 hr. at  $37^{\circ}\text{C}$ . Tissue weights refer to moist tissue pressed between filter paper. Suspending fluid contained  $0.103\text{M}$  NaCl,  $0.031\text{M}$  KCl,  $0.002\text{M}$   $\text{CaCl}_2$ ,  $0.01\text{M}$  glucose, with  $0.025\text{M}$   $\text{NaHCO}_3$  and 5%  $\text{CO}_2$ , in the experiments with brain slices, and  $0.033\text{M}$  phosphate buffer, pH 7.4, in those with uterus slices.

#### OESTRADIOL AND ACETYLCHOLINE IN THE RABBIT UTERUS

As oestradiol appeared to affect neither the breakdown nor the synthesis of acetylcholine, it seemed necessary to repeat the original work of Reynolds in order to see whether we could, in fact, confirm his finding that the acetylcholine content of the intact uterus is increased by oestrogenic stimulation. A total of twenty-six spayed rabbits, comprising twenty Dutch marked and six cross-bred animals were used. The animals were used 10-42 days after spaying and were generally compared in groups of three injected and three uninjected controls. In all but one case, we extracted by our own preferred method, using 3 ml. of 10% trichloroacetic acid and extracting at room temperature [MacIntosh, 1938], but, in order to cover the remote possibility that Reynolds's variation of this method might affect the results, we carried out one test completely as he described [Reynolds, 1939]. Assays were made with the eserinated rectus of the frog [Chang & Gaddum, 1933] or by the blood-pressure of the cat [Brown & Feldberg, 1936] and in one case by both methods together.

The results, listed in Table 2, do not confirm those of Reynolds. By whatever technique the tests were conducted we found about the same amount of acetylcholine in extracts from the uteri of both treated and untreated rabbits, approximating in quantity to that found on the average in Reynolds's tests with oestrogen-treated animals. Only one uterus gave a low figure, a uterus which was heavier than the others and from a control animal. After treatment with cold alkali or incubation with cat plasma, or after atropinization of the cat used for testing, extracts showed 25% or less of the original activity, and the identity of the acetylcholine contained therein was thus reasonably enough established as responsible for the greater part of the activity exhibited. Blank tests on reagents were negative.

The discrepancy between our results and those of Reynolds is, therefore, that we found as much acetylcholine in the uteri of all animals, regardless of

TABLE 2. Acetylcholine content of rabbit uteri

Date of test	Weight of uterus in mg.	Acetylcholine $\mu\text{g./g.}$		Remarks
		Cat B.P.	Frog rectus	
CONTROLS				
29 x 41	280*	0 55	—	*1 horn only
24 xi 41	411	0 55	—	Mean 0 55
	597	0 33	—	
	416	0 77	—	
31 xii 41	456	—	0 43	Mean 0 33 (extraction by Reynolds's method)
	725	—	0 25	
	701	—	0 30	
2 iii 42	227	0 45	—	Used as batch for inactivation tests
	631			
	286			
26 vi 42	750	0 53	0 34	Mean cat 0 30
	1370	0 30	—	
	3800	0 08	0 08	Mean frog 0 21
	Mean	0 445	0 280	
		$\pm 0\ 073\uparrow$	$\pm 0\ 058\uparrow$	
INJECTED				
29 x 41	327*	0 50	—	*Other horn of above after injection
24 xi 41	614	0 49	—	Mean 0 65
	556	0 64	—	
	429	0 83	—	
31 xii 41	514	—	0 22	Mean 0 31 (extraction by Reynolds's method)
	897	—	0 25	
	532	—	0 47	
2 iii 42	270	0 44	—	See above
	254			
	353			
26 vi 42	1050	0 26	0 32	Mean cat 0 37
	1050	0 46	0 23	
	870	0 40	0 34	Mean frog 0 30
	Mean	0 503	0 305	
		$\pm 0\ 059\uparrow$	$\pm 0\ 038\uparrow$	

\* One horn taken before injection, the other 1 hr. after injection of oestradiol.

† Standard error of mean

treatment or procedure, as he found only in those of oestrogen-treated animals. Our results were also less variable than his, within each of the groups compared. The uteri from his rabbits were, in practically all cases, heavier than those from our own (private communication), but it is difficult to see how this can have affected the results, unless, as is hinted by our one heavy control uterus, such uteri have a lower acetylcholine content, which can be increased by oestrogens. If this is the case, it serves to show how rigid must be the conditions under which Reynolds's findings may be obtained, as we have been unsuccessful in attempts to repeat them. However, an examination of Reynolds's original data [1939] shows that a low degree of statistical significance is seemingly to be attached to his results, if our own calculations are correct. We find that the mean value for his controls is  $0.183 \pm 0.120 \mu\text{g./g.}$ , using the data from tests with the eserized frog rectus, the only complete

tests, while that for injected rabbits is  $0.508 \pm 0.125 \mu\text{g./g.}$  The difference is  $0.325 \pm 0.173 \mu\text{g./g.}$  and thus does not exceed twice its own standard error. These calculations disagree with Reynolds's own, published later [Reynolds & Foster, 1939] in which the corresponding means and standard deviations of individual estimates are given as  $0.065 \pm 0.078$  and  $0.420 \pm 0.359$ . From these figures, the difference is  $0.355 \pm 0.101$  and is significant.

### SUMMARY

Attempts to demonstrate an action of oestradiol on choline esterase activity, acetylcholine synthesis, and the acetylcholine content of spayed rabbits' uteri were unsuccessful. It is concluded that, if Reynolds's finding that oestrogens increase the acetylcholine content of the spayed rabbit uterus is to be accepted, it must be recognized that such an increase occurs only under very specific conditions, and that it cannot serve as a general explanation of the vasodilator action of oestrogens on the rabbit uterus.

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## SYNAPTIC POTENTIALS AND TRANSMISSION IN SYMPATHETIC GANGLION

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It has now been established that neuro-muscular transmission is mediated by the local negative potential (the end-plate potential, e.p.p.) which a nerve impulse sets up at the end-plate region of a muscle fibre [Göpfert & Schaefer, 1938; Feng, 1940, 1941; Eccles, Katz & Kuffler, 1941 *a, b*; Eccles & Kuffler, 1941; Kuffler, 1942 *a, b*]. Similarly, it has been shown that synaptic transmission may be mediated by the local negative potential (the synaptic potential) which is set up by a nerve impulse incident on a nerve cell [Gasser & Graham, 1933; Barron & Matthews, 1936; Eccles & Pritchard, 1937; Eccles, 1939].

The curarized nerve-muscle preparation has proved valuable in investigating the end-plate potential, particularly in showing that it is a catelectrotonic potential, and hence relating it to the setting up of muscle impulses. This paper gives an account of similar investigations on the synaptic potentials of sympathetic ganglia. As shown by Langley & Dickinson [1890] synaptic transmission is readily blocked by an intravenous injection of curare. Synaptic potentials alone are then observed, and they have been investigated without the complications introduced by superimposed spike potentials. The cat's stellate ganglion has been chosen for this investigation because the cardiac nerves arise from a fairly homogeneous group of ganglion cells and provide a suitable length of postganglionic nerve for investigation. In addition the work of Bronk and his co-workers [1935, 1938, 1939] gives much valuable information on this ganglion.

### METHOD

The stellate ganglion and its nerves have been exposed through the axilla of the anaesthetized cat (40 mg. nembutal/kg. intraperitoneally) under artificial respiration. Silver-silver-chloride leads have been used for recording. The earth lead forms a ring surrounding the conical projection of the ganglion from which the cardiac nerves arise. The grid lead is via a loop of thread tied to the distal cut end either of the freed cardiac nerves or of the inferior cardiac nerve component alone (cf. diagrammatic representation in Fig. 1*a*). Stimulating electrodes  $S_1$  have been on the

sympathetic trunk posterior to the junction with the 2nd white ramus. Interaction of two preganglionic nerves was studied with electrodes  $S_2$  on the 3rd white ramus, and  $S_3$  on the trunk posterior to the 3rd ramus connexion. The ganglion is dissected just sufficiently to allow the earth lead to surround the postganglionic origin without making contact with surrounding tissues. All its preganglionic supply is cut centrally, but its blood supply is preserved as much as possible.

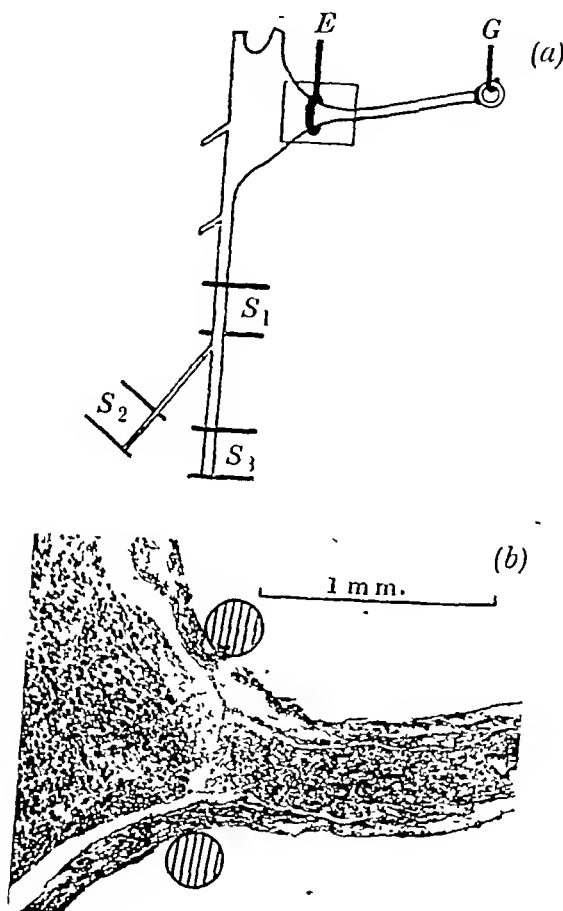


Fig. 1. (a) Sketch of stellate ganglion showing position of recording ( $E$ , earth and  $G$ , grid) and stimulating electrodes. (b) Microphotograph of section included in the rectangle of (a). The two circles show in section the position and approximate size of the silver ring, which is seen to lie slightly beyond the sharp boundary between the ganglion cells and the cardiac nerve. A connective tissue sheath surrounds both the ganglion and the nerve.

The stimulating and recording apparatus and the moist warm preparation chamber have already been described [Eccles & O'Connor, 1939]. A four-stage negative feed-back amplifier is used giving an output potential strictly proportional to input for outputs as high as 100 V. Large coupling condensers give it a time constant of 6 sec., which was large enough to prevent serious distortion of all potentials described in this paper. Maximum repetitive stimulation at varying frequencies and durations is provided by a thyratron stimulator.

Merck, Inc., U.S.A., kindly donated the very high potency curare used throughout this investigation. Intravenous injection of 1 mg./kg. (extremes  $\frac{1}{2}$ –2 mg./kg.) was usually adequate to block synaptic transmission through the ganglion. The onset of the curare paralysis is fairly rapid—maximum effect is observed just over  $\frac{1}{2}$  min. after the injection. Recovery begins 1–1½ min. later, and half recovery occurs in 10–20 min.

## RESULTS

(1) *The synaptic potential*

When sufficient curare is injected intravenously, the transmission of impulses through the stellate ganglion is blocked for some minutes. However, a pre-ganglionic volley still causes the ganglion to become electrically negative to

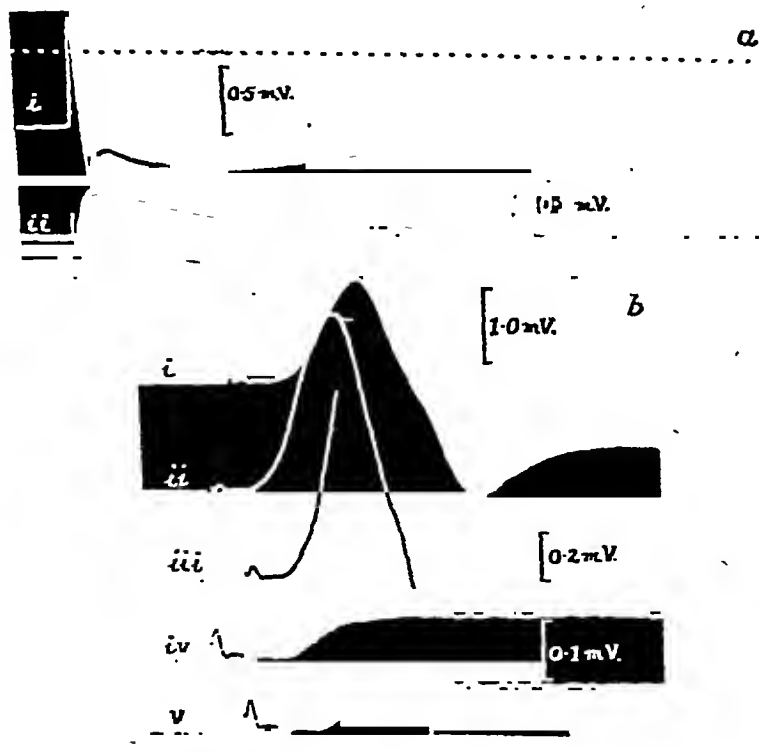


Fig. 2. (a) Action potentials from stellate ganglion, (i) before and (ii) after complete curarization. Time = 10 msec. (b) As in (a), but at faster speed; (i), (ii) and (iii) before curarization at progressively increasing amplification to show double-step rise. (iv) and (v) after complete curarization and at still higher amplification. Note the lengthened latent period. Time line shows 10 msec. Voltage scales shown for (i), (iii), (iv) and (v).

the isolated postganglionic trunk (Figs. 2 a(ii), 3 a, 4 a). This potential change is easily distinguishable from that due to postganglionic nerve impulses (Fig. 2 a(i)): (i) it shows rapid decrement as the recording electrode is moved away from the ganglion; (ii) it has a much slower time course.

The decremental spread has been approximately determined by placing the recording electrode at different positions along the postganglionic trunk, the reference electrode being on the isolated end of the trunk. In this way the negative potential is observed to fall to  $1/\epsilon$  in about 1.7 mm. For the postganglionic trunk of the superior cervical ganglion a fall to  $1/\epsilon$  in 2-2.5 mm. was found for the slow positive potential wave [Eccles, 1935*b*]. Histological examination has shown that this decremental spread is not associated with a scattering of ganglion cells along the postganglionic trunk (cf. Fig. 1*b*). The decremental spread in fact is a typical electrotonic phenomenon. The ganglion cells become electrically negative to their axons (the postganglionic fibres), and this potential spreads by local current flow along the axons to give an approximately exponential spatial decrement. In curarized muscles the end-plate potential was similarly observed to spread electrotonically from the end-plate region along the muscle fibres [Eccles *et al.* 1941 *a, b*]. The closely analogous negative potential of the curarized ganglion must be set up by the pre-ganglionic volley primarily in the immediate vicinity of the preganglionic endings, i.e. at the synaptic regions of the ganglion cells, and it may therefore be called the synaptic potential.

With the neuro-muscular junction a pure end-plate potential is also set up by a second nerve volley acting while the muscle is still relatively refractory after its response to the first volley [Eccles & O'Connor, 1939; Eccles & Kuffler, 1941]. Occasionally small synaptic potentials have been observed with a similar technique, but usually the earliest second preganglionic volleys also set up the discharge of impulses from the ganglion.

## (2) *Size of the synaptic potential*

As shown in Fig. 1*a* the reference electrode is on the isolated end of the cardiac nerve, while the leading electrode is a ring of chlorided silver wire through which the postganglionic pole of the ganglion is drawn. The effective leading point of this latter electrode will be at the region of abrupt diminution of cross-sectional area which occurs as the postganglionic pole of the ganglion gives origin to the cardiac nerve (cf. Fig. 1*b*). Potentials arising in a ganglion cell will only be recorded after electrotonic transmission along its postganglionic fibre to this region, and will consequently suffer a diminution of size and a slowing of time course. However, most of the ganglion cells belonging to the cardiac nerve presumably are situated in the large postganglionic pole from which this nerve usually arises (Fig. 1*b*), hence their potential changes will be recorded with relatively little diminution or delay. In a few preparations there has been no postganglionic pole, the cardiac nerve arising abruptly from the ganglion. The very small synaptic potentials then observed possibly may be attributed to the large decrement involved in the electrotonic spread from

ganglion cells buried deeply in the ganglion at some distance from the origin of their postganglionic trunk.

(3) *Time course of synaptic potential* (cf. Figs. 2a, 2b, 3a, 4a)

*Latent period.* There is a latent period of about 5 msec. (extremes 3.8 and 7.5 msec.) between the preganglionic stimulus and the beginning of the synaptic potential. Preganglionic conduction for about 10  $\mu$ m. would account for only about 1 msec. of this, though conduction in the fine preganglionic terminals may appreciably add to this time. Thus the true synaptic delay would appear usually to be about 3-4 msec.

*Rising phase and summit.* Initially the potential rise is fairly rapid ( $\frac{1}{2}$  in 2.5-4.5 msec.), but it then slows to reach very gradually a flattened summit usually at 10-20 msec. from its origin.

*Falling phase.* Decline from the flattened summit is slow at first, half decay being reached at 60-90 msec. from the summit; but thereafter it is approximately exponential, halving occurring in 40-60 msec. ( $1/e$  in 60-85 msec.).

Further, curare in excess of that required to block synaptic transmission diminishes the synaptic potential, but does not significantly alter its latent period or time course (cf. Fig. 3a(i), (ii)). In some preparations with apparently complete curarization, a small spike may still be superimposed on the synaptic potential, and its removal by deeper curarization may simulate a delay in spike summit.

The whole time course is thus much slower than the end-plate potential in cat's muscle [cf. Eccles *et al.* 1941b, Table 1). The latent period is about 5-7 times longer, the rising phase initially about 10 times slower, and the summit-time and rate of decay 15-20 times slower.

(4) *Relation of synaptic potential to the initiation of nerve impulses*

As curarization passes off, the synaptic potential shows at first an increase in size but no alteration in time course. Then at a critical size a small spike arises from its summit and is propagated postganglionically (Fig. 3a(iii)). Synaptic transmission has been restored for a few ganglion cells. With further recovery the spike is larger, takes off progressively earlier from the synaptic potential, and seems eventually to submerge it completely, no initial phase of synaptic potential being apparent.

In fourteen ganglia the size of the synaptic potential has been measured for critical curarization, i.e. at the point where impulse transmission is just blocked. In five it was as high as 10-12 % of the maximum spike potential recorded from the normal ganglion. Seven gave figures of 5-8 %, and the remaining two with less than 3 % showed the abrupt origin of the postganglionic trunk from the ganglion. The highest values (10-12 %) presumably give the best indication of the ratio, threshold level of synaptic potential to

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below about 20 % of the spike potential, no impulse is set up—synaptic transmission is blocked.

An attempt to investigate the relationship of synaptic potential to spike has been made in seven experiments by means of the detailed matching of the initial foot of the normal or partly curarized potential with the pure synaptic potential obtained after complete curarization [cf. the matching technique with the end-plate potential-muscle spike relationship, Eccles & Kuffler, 1941]. In six, matching was only possible for a very small part of the foot (less than 3 % of the spike height in the curare-free ganglion). The spike appeared to arise when the synaptic potential was very small [cf. some soleus strip preparations, Eccles & Kuffler, 1941]; nevertheless, when completely curarized, the synaptic potential may be large (up to 12 % of the spike potential). In the other experiment a single preganglionic volley set up a double step potential (Fig. 2*b*), and the matching suggested that the spike arose from an initial step (12 % of the spike height) of synaptic potential [cf. some soleus strip experiments, Eccles & Kuffler, 1941, Figs. 3, 4]. In all, curarization is associated with a considerable lengthening of latent period, 2.8 msec. in Fig. 2*b*. This effect contrasts with the action of curare on neuro-muscular transmission where the lengthening of latency of the end-plate potential was often undetectable and never more than 0.1 msec. [Eccles & Kuffler, 1941; Kuffler, 1942*a*].

#### (5) *Summation of synaptic potentials*

(*a*) *Double nerve volleys.* In a curarized ganglion a second preganglionic volley adds its synaptic potential on top of the potential produced by the first volley (Fig. 4*a*). The synaptic potential produced by the second volley has been determined by subtracting the potential set up by the first volley alone from the summed potential as was done for the end-plate potential [cf. Eccles *et al.* 1941*b*]. With all volley intervals the time courses of the second synaptic potentials thus determined have not differed appreciably from the first, but they usually have been about 15–30 % larger (cf. Fig. 4*a*) for volley intervals up to 0.2 sec. However, in one experiment the potentiation of the second response was much greater—100 % larger at intervals of a few milliseconds (Fig. 3*b*(i), (ii)), progressively falling with lengthening interval to about 50 % larger at 0.2 sec. Similar large potentiations occur with successive end-plate potentials in frog's muscles [Feng, 1940; Eccles *et al.* 1941*b*]. With another ganglion the synaptic potential of the second volley was slightly smaller than the first (about 80 %), thus resembling the end-plate potentials of cat's muscle [Eccles *et al.* 1941*b*].

(*b*) *Repetitive stimulation.* With repetitive stimulation at very slow rates (5 a second or less) each successive synaptic potential is practically independent of the preceding. It is superimposed on a negligible residuum of the preceding potentials, and its size and time course differ but little from the initial single

spike potential, for an individual ganglion cell. With the end-plate potential, ratios up to 8 % were recorded with multifibre preparations [Eccles *et al.* 1941*b*], but, with the isolated single neuro-muscular junction, threshold values ranging from 30 to 35 % were observed with critical curarization [Kuffler, 1942*a*]. It is possible that these values for the isolated junction are higher than obtain in the intact preparation because in the latter (see Discussion) the

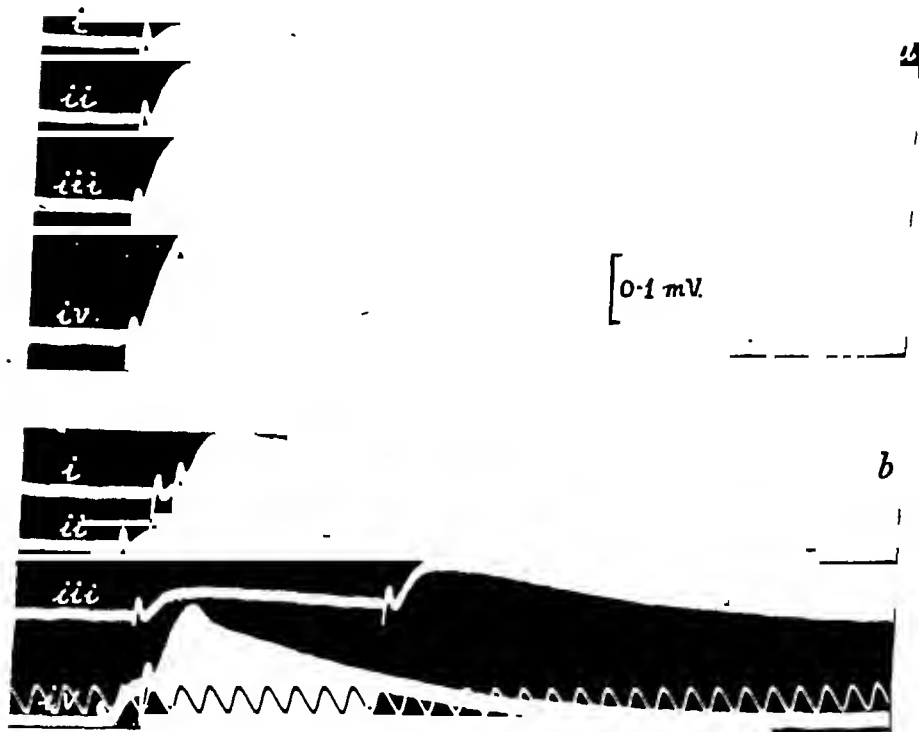


Fig 3 (a) Action potentials from stellate ganglion showing gradual recovery from the deep curarization of observation (i) Spike is first shown in (ii) and is large in (iv) (b) As in (a), but showing response to two preganglionic volleys in (i), (ii) and (iv) Curarization is less with (iv) and summation gives a spike resembling that of a (iv), though the summed end-plate potential is much greater.

currents set up by end-plate potentials in adjacent junctions would lower threshold in the same way as occurs at the cathode of an applied current pulse [Katz, 1939*a*]. On the other hand, in the multifibre preparations scatter of the endings with the consequent electrotonic decrement will cause the observed ratio to be lower than that actually obtaining at a single junction. It would appear that a value of about 20 % gives the order of magnitude of the ratio for both ganglion cells and muscle fibres in the intact preparation, i.e. when curare depresses the local potential produced by presynaptic



and 140 a second responses. However, in its latter part the decay is almost as fast (halving in 50–60 msec.), as with responses to single stimuli or slow

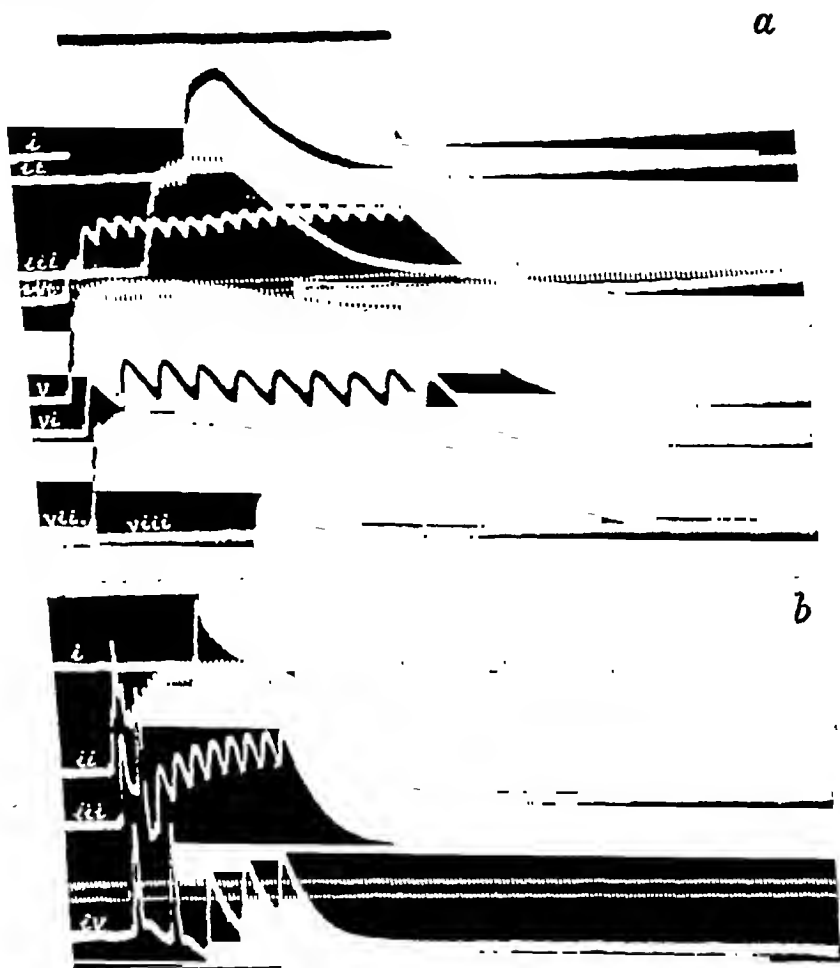


Fig. 5. (a) Action potentials of stellate ganglion. (i) calibrating rectangular potential of 0.4 mV. 0.7 sec. duration applied in series with the preparation. With (ii)–(vii) repetitive preganglionic stimulation: 140 a sec. for 0.1 sec.; 84 a sec. for 0.2 sec.; then 28, 84, 13.5, and 140 a sec. for 0.7 sec.; (viii) single response. Time = 10 msec. (b) As in (a) but with less curarization. (i) Single response: (ii), (iii), (iv) 84, 28 and 13.5 a sec. for 0.4 sec. Time = 10 msec. Potential scale as for (a).

rates of stimulation. With shorter durations of the high-frequency stimulation this initially slowed decay is much less marked (observations (ii) and (iii)), and with longer durations it is still slower. This initial period of slowed decay is always evident after repetitive stimulation by more than 15 stimuli at rates above 50 a second.

response. With faster rates the response to each successive volley is modified in two ways (cf. Fig. 5*a* at 13.5 and 28 a sec.): (i) it is superimposed on a residuum of the preceding synaptic potentials; (ii) after the usual small potentiation during the first few responses it is smaller than the initial single response.

With moderate rates of stimulation (for example at 28 a sec. in Fig. 5*a*) there is, as a consequence, a stepped rise to a wavy plateau, which is well maintained even during several seconds' stimulation. The plateau is attained

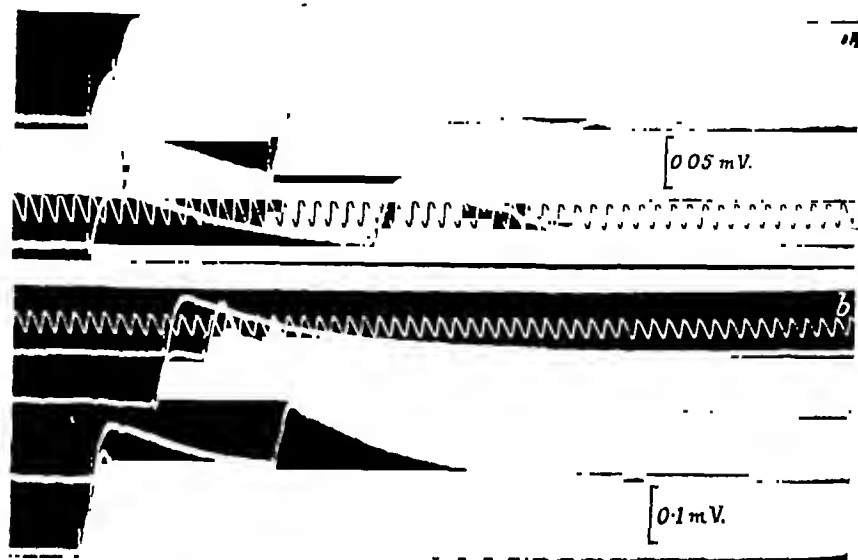


Fig. 4. (*a*) Summation of action potentials set up by two preganglionic volleys in curarized ganglion. (*b*) As with (*a*), but with less curarization, showing facilitation resulting from summation. Even the single responses probably have a small superimposed spike. Time = 10 msec. The small waves on the declining phase of many records of Fig. 4 (cf. also Fig. 2*a*(ii)) are heart action potentials.

earlier and is higher, the faster the stimulus frequency. At the end of the stimulation the summed synaptic potential decays at a rate but little slower than that observed for the single response.

With higher rates of stimulation, e.g. 84 and 140 a second in Fig. 5*a*, the plateau is still higher and shows little or no sign of undulations corresponding to the stimulus frequency. It is usually about three times the single synaptic potential, but ratios as high as five are observed in the more deeply curarized preparation. This plateau slowly declines after about 0.2 sec. (observations (v) and (vii), Fig. 5*a*), and after 1–2 sec. stimulation becomes stabilized at a lower level. Observations (v) and (vii) Fig. 5*a* show that at cessation of stimulation the synaptic potential initially decays at a much slower rate than normal, e.g. half decay takes respectively about 150 and 200 msec. with the 84

this supernormality is small and its facilitating action is offset by accommodation (cf. above). The facilitation curve is thus largely determined by factor (i) above, as has already been observed for the *N* wave (the synaptic potential) and the facilitation curve with submaximal preganglionic volleys [Eccles, 1935*b*, Text-fig. 19]. In the experiment with a subnormal second synaptic potential the facilitation curve was much shorter than the synaptic potential, quickly passing over to a subnormal spike response with volley intervals longer than 80-100 msec.

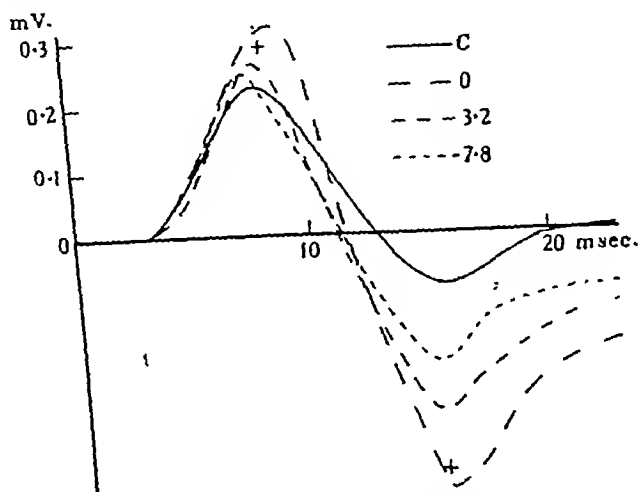


Fig. 6. Single submaximal stimuli have been applied through electrodes  $S_1$  and  $S_2$  of Fig. 1. The continuous line shows ganglion spike potential produced by  $S_1$  alone. The other three curves show the spike potentials added by  $S_2$  to the  $S_1$  response when set up simultaneously with  $S_1$  or 3.2 or 7.8 msec. later than it. The crest and trough positions with stimuli at  $S_2$  1.6 msec.  $S_1$  are shown by crosses and are transitional between the 0 and 3.2 msec. intervals.  $S_1$  stimulus at zero on time scale.

With curare-free stellate ganglia, facilitation phenomena are only observed with submaximal preganglionic volleys and then have closely resembled those observed for the homologous  $S_2$  cells in the superior cervical ganglion.

(i) With both stimuli to the same preganglionic nerve, there is either facilitation with shorter intervals, which passes over to inhibition with longer intervals [cf. Eccles, 1935*a*, Text-fig. 3], or the facilitation may be submerged beneath the inhibition at all intervals. The time course of this combined effect closely resembles the summed effect of the slow negative potential and the more prolonged slow positive potential [cf. the superior cervical ganglion, Eccles, 1935*b*, Text-fig. 20; the inferior mesenteric ganglion, Lloyd 1939*a*, *b*].

(ii) This prolonged facilitation-depression effect may also be observed when the two stimuli are applied to separate preganglionic nerves. However, it

The repetitive synaptic potentials usually decay to a small after-positivity (cf. Fig. 5*a*). This after-positivity is much smaller for the calibration potential applied in series with the preparation (Fig. 5*a*(i)), hence presumably it is largely physiological. It may be the slow positive potential set up by spike responses which are too small and asynchronous to give noticeable irregularities on the synaptic potential record. Alternatively, the synaptic potential itself may set up a small after-positivity of the ganglion cells resembling that set up by a propagated impulse, either dromic or antidromic [Eccles, 1935*b*, 1936; Lloyd, 1939*b*].

(*c*) *Synaptic facilitation.* When the degree of curarization is less than in Fig. 4*a*, summation of the two synaptic potentials causes the initiation of propagated spikes (Fig. 4*b*). Synaptic transmission occurs with two volleys, but not with one alone, i.e. there is synaptic facilitation. However, facilitation is not solely conditioned by summation, for the threshold value of the summed synaptic potential is higher than that observed for a single volley when the curarization is diminished (cf. Fig. 3*b*(iv) with Fig. 3*a*(iv)). No such effect suggesting accommodation was observed with summing of end-plate potentials to give neuro-muscular facilitation [Eccles *et al.* 1941*b*], a difference which may perhaps be correlated with the much slower accommodative process in muscle [Lucas, 1907; Blair, 1941]. Accommodation is still more evident with repetitive stimulation. Thus in Fig. 5*a* the synaptic potentials were built up to values much higher than the spike threshold for a single volley (cf. observation (i), Fig. 5*b*), and yet no spikes were set up. This effect is still more evident in Fig. 5*b*, where the curarization had partly worn off. The first two or three volleys set up large spikes, but even at slow rates of stimulation only synaptic potentials are set up by the later volleys. Presumably the slow positive potential (cf. the early positive dip in observations (ii), (iii), (iv), Fig. 5*b*) resulting from the spike response of some ganglion cells inhibits further discharge of impulses by those cells [cf. Eccles, 1935*b*; Lloyd, 1939*a, b*; Bronk, 1939]. However, accommodation must also play a part in preventing the high levels of synaptic potentials from setting up spikes in the latter parts of observations (ii), (iii), (iv) of Fig. 5*b*.

In Fig. 4*b* the facilitated spike is just detectable at the longest volley interval, and is larger at the shorter volley intervals, where there is increased summation of the two synaptic potentials, i.e. the higher the synaptic potential, the more ganglion cells excited above threshold. A facilitation curve could be constructed by plotting the spike size against the volley interval, as has been done for excitation of the superior cervical ganglion by submaximal pre-ganglionic volleys [Eccles, 1935*a*]. However, just as with neuro-muscular facilitation, the facilitation curve depends upon two factors: (i) the size and time course of decay of the first synaptic potential on the declining remainder of which the second synaptic potential is added; (ii) the supernormal or subnormal size of the second end-plate potential. The usual supernormal effect outlasts the single synaptic potential; hence the facilitation curve should be more prolonged than the synaptic potential. However, in most experiments

(2) *The time course of the transmitter action*

Since the latter part of the synaptic potential is a period of passive electrotonic decay with an approximately exponential time course, it can be analysed on the basis of Hill's 'local potential' theory [1936] in order to obtain the probable time course of the active depolarizing agent (the transmitter action). An analysis of this kind [Eccles *et al.* 1941*b*, equation (1) and Figs. 19, 20] gave a time course for the transmitter action at the frog's neuro-muscular junction, which corresponded well with more direct determinations on the isolated single nerve-muscle junction [Kuffler, 1942*b*].

Several errors are introduced by this analysis [cf. Eccles *et al.* 1941*b*], but they are small relative to the comparatively gross effects discussed below, and, if allowed for, would actually accentuate these effects.

(i) The end-plate potential of curarized muscle is associated with a lowering of impedance at the junctional region [Katz, 1942], which in part is due to the catelectrotonus, and in part seems to be due to a direct action of the transmitter. Presumably this lowered impedance would also occur with the synaptic potential, and there would be an associated shortening of time constant not allowed for in the analysis. As a consequence, in Figs. 7 and 8, the peak of the transmitter action should be a little higher, and the initial part of the tail should be slightly raised relative to the later part.

(ii) On account both of the electrotonic spread of the recorded synaptic potentials and of the variations in latencies of the individual component responses, the synaptic potentials for a single ganglion cell will have a quicker rising phase and a slightly quicker falling phase than the recorded aggregate response. The individual cells would thus exhibit a sharper and higher peak relative to the tail than shown in Figs. 7 and 8.

Fig. 7 shows that, as determined by the above analysis, the depolarizing agent shows a very brief high intensity action, reaching its maximum in about

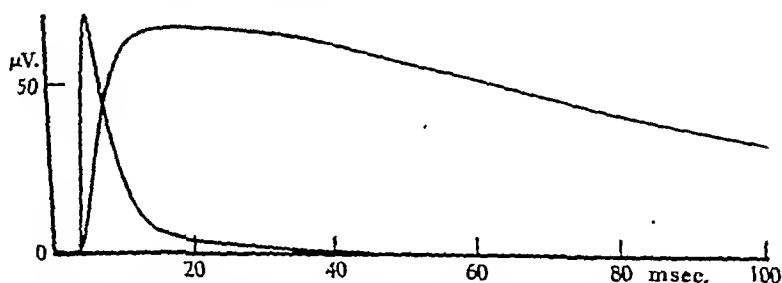


Fig. 7. Single synaptic potential from experiment of Fig. 3 plotted together with time course of transmitter action determined by analysis as described in text. Stimulus at zero on time scale. Ordinates for transmitter action are in arbitrary units.

2.5 msec., and decaying to one-tenth in 12 msec. from its origin. Thereafter its decay is much slower, but it has practically disappeared at 40 msec. The extreme values in our experiments have been 2–3.5 msec. for the time to maximum, 10–18 msec. for decay to one-tenth, and no transmitter action has been detectable after 50 msec.

often cannot be detected, presumably because the respective fields of ganglion cells do not overlap sufficiently.

(iii) With stimuli to separate preganglionic nerves a separate brief facilitation effect has been observed with very short stimulus intervals in four out of ten experiments (Fig. 6). Its time course corresponds with that observed for  $S_1$  ganglion cells [Eccles, 1937, Fig. 2] if allowance be made for the slower time course of all  $S_2$  cell reactions. When the ganglion is partly curarized, this initial brief facilitation is not enhanced, but it remains distinct from the later increased facilitation which runs the slow time course that associates it with summation of synaptic potentials. Finally, when curarization is paralytic or nearly so, the facilitation at short intervals is not separable from that at long intervals. The maximum at short intervals then declines progressively as the stimulus interval lengthens, giving with all intervals the facilitation that would be expected on the basis of summation of synaptic potentials.

## DISCUSSION

### (1) *The nature of the synaptic potential*

In a curarized ganglion a preganglionic volley has been shown to set up a local negative potential, the synaptic potential. The following evidence identifies this with the catelectrotonic potential set up by a subthreshold electric stimulus, and thus shows it to be analogous to the end-plate potential produced at curarized neuro-muscular junctions.

(1) The synaptic potential spreads decrementally along the postganglionic trunk, halving in about 1.2 mm., and with a progressive slowing in its time course.

(2) The latter part of the synaptic potential decays approximately exponentially, falling to  $1/e$  in 60–80 msec.

(3) If the synaptic potential set up by a single volley exceeds a certain critical level, an impulse is initiated by the ganglion cell.

(4) There is summation of synaptic potentials set up by successive preganglionic volleys. The resulting increased potential is the basis of synaptic facilitation. However, a continued synaptic potential is associated with a progressive increase in the threshold level at which spikes arise. This effect parallels the accommodation occurring when a catelectrotonus is set up by application of a continued electric current to nerve [Hill, 1936; Katz, 1939b].

It has been impossible to apply the other tests used with the end-plate potential because they depend on interaction with an antidromic volley. The slow negative and positive potentials set up by an antidromic volley [Eccles, 1936; Lloyd, 1939b] resemble those arising with preganglionic stimulation of the ganglion and obscure any effects that the antidromic spike has on the synaptic potential.

(i) During repetitive stimulation at any frequency there is a progressive lowering of the peak and increase in the tail (cf. Fig. 8c, 85 a sec. for 0.2 and 0.7 sec.; Fig. 8d, 140 a sec. for 0.1 and 0.7 sec.); however, the successive additions to the tail resemble the peak in showing a progressive decline.

(ii) When the number of stimuli is approximately constant, the faster the rate (within limits) the lower is the peak and the larger the tail (cf. Fig. 8b, 19 stimuli at 55 a sec. with 20 stimuli at 28 a sec., also with Fig. 8c, 17 stimuli at 84 a sec.): with the faster frequencies there is more overlapping, and hence summation, of the tails produced by successive stimuli.

Thus the tail behaves exactly as would be expected for a slowly decaying fraction of the transmitter. Little of this fraction appears after a single volley, but, on account of its slow decay, it is built up by successive volleys, particularly at frequencies over 50 a sec. The fraction responsible for the initial peak gives evidence by its size of the rapid decline in the quantity of transmitter produced by successive volleys at high frequency. Since there is an approximate correlation between this decline and the decline in the successive additions to the tail, there is no evidence that the peak and the tail are produced by two independent depolarizing agents. The results conform well with the postulate of a single depolarizing agent which is produced in successively smaller quantities during repetitive stimulation, this decline being more rapid the higher the frequency; and which may be removed by either a quick or a slow process.

The decline in the production of depolarizing agent during repetitive stimulation is also well shown by the end-plate potential at the curarized neuromuscular junction [Feng, 1940; Eccles *et al.* 1942]. Moreover, the action of eserine reveals that there is both a quick and a slow process for removal of the neuro-muscular transmitter [Eccles *et al.* 1942]. The light which the above findings throw on the nature of the synaptic transmitter will be considered in a later paper describing the action of eserine on synaptic potentials.

### (3) *The relation of the synaptic potential to synaptic transmission in the curare-free ganglion*

With curarized ganglia there would appear to be no doubt that the synaptic potential is entirely responsible for the synaptic transmission that occurs on account of the summation of successive volleys, or when the synaptic potential increases as the curare block passes off. With curare-free or even with partly curarized ganglia the position is obscure for two reasons: (a) in only one experiment (with the double-step potential rise) has it been possible to demonstrate a large synaptic potential preceding the discharge of impulses from the ganglion; (b) with interaction between submaximal volleys in two separate preganglionic nerves there may be a brief phase of facilitation separated by

After repetitive stimulation the latter parts of the synaptic potential decayed almost as rapidly as with the responses to single volleys (Fig. 5*a*). Presumably, therefore, the decay is then largely passive, and no large error will be introduced by assuming that the electrotonic time constant is the same as with the single-volley response. On this basis the potentials following cessation of repetitive stimulation in Fig. 5*a* have been analysed and plotted in Fig. 8.

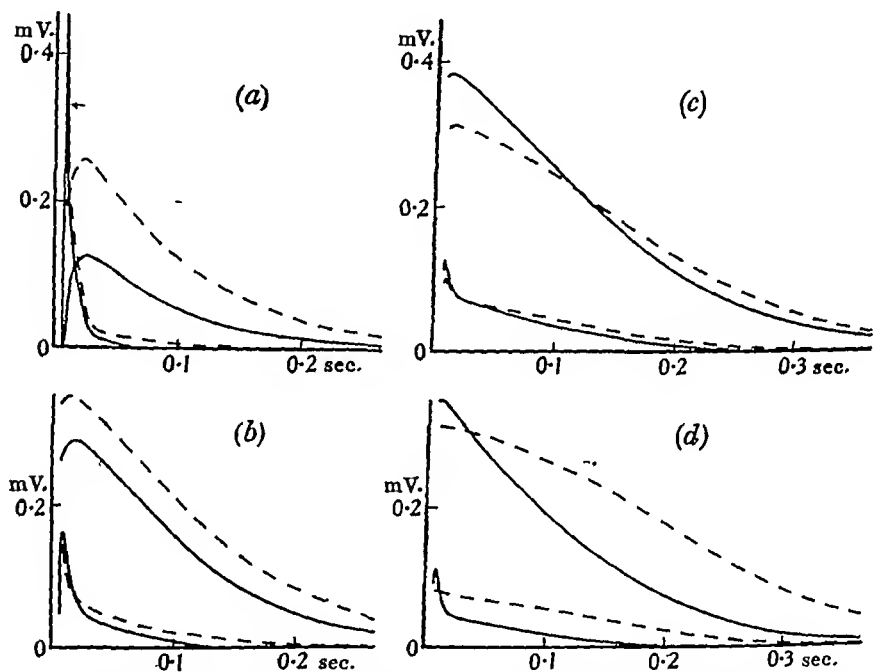


Fig. 8. Plottings and analyses as in Fig. 7, but for repetitive stimulation, all observations but one being illustrated in Fig. 5*a*. The plottings have been made relative to the steady base-line to which the potential decays at the end of stimulation (usually slightly positive to the initial base line). (a) shows plots and analyses of single stimulus (continuous lines) and 13.5 a sec. for 0.7 sec. (broken lines), the summit level of latter analysed curve being shown by arrow; (b) 28 a sec. for 0.7 sec. (continuous lines) and 55 a sec. for 0.35 sec. (broken lines); (c) 84 a sec. for 0.2 sec. (continuous lines) and 0.7 sec. (broken lines), (d) 140 a sec. for 0.1 sec. (continuous lines) and 0.7 sec. (broken lines). Zero time corresponds to last stimulus of the repetitive series. All analysed curves are plotted on same arbitrary ordinate scale.

In general it will be seen that, just as with the single-volley response (Figs. 7, 8*a*), the transmitter action shows an initial sharp peak (except with 140 a sec. for 0.7 sec.) and a later slow tail. The time course of the initial peak resembles that for a single response, but it is smaller and the tail is larger and longer. Fig. 8 further illustrates the following general observations in regard to the transmitter action with repetitive stimulation.



postulating a special detonator action. The flow of current from active nerve fibres and cells has been shown to play an important part in modifying the excitability of adjacent fibres and cells [Katz & Schmitt, 1939, 1942; Renshaw, 1941]. When a ganglion is excited by a preganglionic volley, there are two sources for currents which could act on those ganglion cells not discharging impulses: (i) the spike potentials of preganglionic fibres in close proximity to that ganglion cell; (ii) the potentials arising in adjacent activated ganglion cells—synaptic potentials in those subliminally excited, and in addition spike potentials with the later slow positive wave in those discharging impulses. It seems likely that the current flowing when ganglion cells are negative to their axons during either a synaptic potential or the discharge of an impulse would act on adjacent cells as a local cathode. With impulses this stimulating action would have approximately the time course of the brief facilitation of Fig. 6. It should be pointed out that the local origin of these negative potentials (ganglionic spikes or synaptic potentials) would distinguish their effect from that produced by propagated spikes, where the initial effect is anodal [Katz & Schmitt, 1939]. It is possible also that the brief facilitation may be caused by summation of the brief depolarizing actions at adjacent synapses.

Again, since the currents generated by synaptic potentials in adjacent cells would lower the threshold in any cell, they would cause the spike to be initiated at a lower value of synaptic potential, and this effect would be increased by the earliest spikes similarly acting on adjacent cells. Thus the initial step of synaptic potential would be diminished for the individual ganglion cell, and with the aggregate potential recorded from the intact ganglia it would suffer further reduction on account of the temporal dispersion of the individual responses. Until these factors are evaluated, no theoretical significance should be attached to the spike origin at very low values of the synaptic potential.

Thus it may be provisionally concluded that the initiation of impulses in ganglion cells can be regarded as mediated by the known factors; principally the brief active depolarizing agent setting up the synaptic potential, but also the accessory currents generated in adjacent cells and nerve fibres; there is no need to postulate a special brief excitatory mechanism, the so-called detonator response.

#### SUMMARY

When synaptic transmission through a sympathetic ganglion is blocked by curare, a preganglionic volley sets up a local negative potential of the ganglion cells relative to their axons—the synaptic potential.

This potential spreads decrementally (halving in about 1.2 mm.) along the postganglionic fibres. After an initial rapid rise it reaches a flat-topped summit at 10–20 msec. and decays slowly—in its latter part approximately exponentially with successive half times of 40–60 msec.

a trough from the facilitation that runs the long time course characteristic of summation of synaptic potentials.

This brief facilitation has been previously observed in the central nervous system [Lorente de Nó, 1935, 1939] and in the superior cervical ganglion [Eccles, 1937], and it has been suggested that it is due to summation of a brief excitatory process (the detonator response) which is the normal exciting mechanism in synaptic transmission. In addition to the brief initial phase of facilitation, the concept of the detonator response was based on the following evidence [Eccles, 1937].

(i) The short and uniform duration of the synaptic delay for one type of ganglion cell—particularly the  $S_1$  ganglion cell—even when it is excited to discharge by a just-threshold stimulus. (ii) This synaptic delay can only be slightly lengthened (about 2 msec.) by a preceding antidromic volley. Further retardation of the antidromic volley prevents synaptic transmission altogether. These two evidences for brevity of action were contrasted with the slow time course of the synaptic potential (*N* wave), which took 10–15 msec. to reach its summit.

The conditions obtaining with the antidromic interaction ((ii) above) have recently been investigated in the isolated single neuro-muscular junction [Kuffler, 1942*b*]. There it has been shown that the end-plate potential cannot be superimposed on the antidromic spike potential. It is completely destroyed by the spike, and after the spike it can be built up only by the action of any surviving depolarizing agent. If similar conditions obtain with the sympathetic ganglion, the antidromic interaction ((ii) above) shows that the active depolarizing agent cannot produce a threshold depolarization if the antidromic spike prevents it from acting for about 2 msec. The brief time course of action revealed by analysis (Fig. 7) agrees well with this conclusion—particularly as the value of 2 msec. was obtained for the much faster  $S_1$  ganglion cells.

The short and uniform duration of the synaptic delay ((i) above) may also be correlated with the brief time course of the depolarizing agent, for this causes the steep initial rise of the synaptic potential (cf. Fig. 7), and, if an impulse is not set up during this steep rise, the accommodation process will tend to prevent it arising during the flattened summit of the synaptic potential. The above two lines of evidence may therefore be related to the short duration of the active depolarizing agent, and not to a separate hypothetical detonator action.

Thus the evidence for a separate detonator action is reduced to the brief phase of facilitation (cf. Fig. 6), and the finding that the spike often arises when the synaptic potential is very small (§ 4), as is also observed with the end-plate potential in some soleus preparations [Eccles & Kuffler, 1941]. It now seems probable that these lines of evidence can be explained without

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At just complete curarization the synaptic potential may be as high as 12 % of the spike potential, and with still less curarization spikes are superimposed on the rising phase.

In all these respects the synaptic potential resembles a catelectrotonic potential and thus is analogous to the end-plate potential of curarized muscle.

There is summation of the synaptic potentials set up by two preganglionic volleys, the second being usually about 20 % larger than the first. If the summed potential is high enough, the ganglion cells discharge impulses. This facilitation has a similar time course to the synaptic potential.

Summation also occurs with repetitive stimulation, but an accommodation-like process then cuts short the facilitation.

Synaptic potentials set up by single or repetitive stimulation have been analysed on the basis of Hill's local potential theory. As determined in this way the synaptic transmitter action exhibits a brief high intensity phase (peak in about 3 msec., 10–18 msec. total duration) followed by a prolonged low phase. With rapid repetitive stimulation the prolonged phase is greatly increased and lengthened by summation, and after 100 stimuli may persist for as long as 300 msec. These results may be reconciled with the existence of a single synaptic transmitter by postulating both a quick and a slow process for its removal.

The processes involved in synaptic transmission and facilitation are discussed, particularly the brief so-called detonator facilitation, and it is concluded that most and possibly all of the evidence for the detonator action may now be attributed to the brief transmitter action.

The author wishes to thank the National Health and Medical Research Council of Australia for equipping and maintaining the workshop in which most of the apparatus was made, and also Merck, Inc., U.S.A., for kindly donating the very high potency curare that was used throughout this investigation.

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 Eccles, J. C. & Kuffler, S. W. [1941]. *J. Neurophysiol.* **4**, 402.

After closure of the abdomen, the animal was allowed to survive for a period thought to be sufficient to allow absorption of anything from  $\frac{1}{2}$  to  $\frac{3}{4}$  of the salt in the gut lumen. At the end of this period the cat was killed, the intestine removed, and the gut contents recovered by washing through with 1 l. warm water. The mandelic acid was extracted with ether and the optical activity determined in acetone as previously described [Garry & Smith, 1940]. The extracted material, without further purification, showed, on analysis and on determination of the equivalent, very satisfactory agreement with the theoretical values for pure mandelic acid.

In decerebrated and in anaesthetized cats urine was secreted during the absorption period. The urine was collected through a cannula in the urinary bladder and the mandelic acid extracted and examined.

Since it has been claimed that the gut *in vitro* shows powers of selective absorption [Auchinachie, Macleod & Magee, 1930; Macleod, Magee & Purves, 1930], we examined the behaviour of sodium *dl*-mandelate solutions when in the small intestine of the cat *in vitro*. The *in vitro* technique is illustrated in Fig. 1.

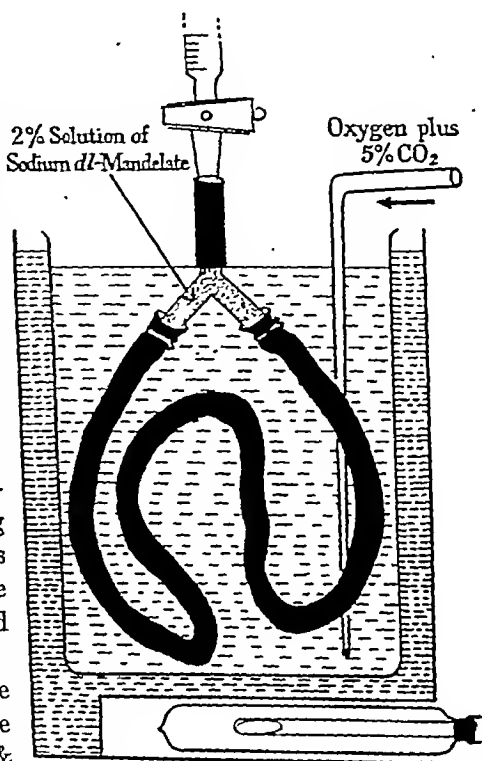


Fig. 1. Entire small intestine of cat *in vitro*. Active circulation of the gut contents takes place.

## RESULTS

*Decerebrated cats.* Absorption of sodium mandelate from the small intestine was rapid. In 1 hr. roughly  $\frac{3}{4}$  was absorbed, practically all being removed in 2 hr. The mandelic acid, recovered from the residue in the gut, invariably showed obvious, although not marked, dextro-rotation. A typical result is shown in Table 1 (a).

Absorption from the colon was very much slower, but again there was a preponderance of the *d*-form of the acid left in the gut (Table 1 (b)).

The urine, on the other hand, contained mandelic acid with quite pronounced laevo-rotation (Table 1).

*Anaesthetized cats.* Although sodium *dl*-mandelate disappeared rapidly from the small intestine when the cats were kept continuously under the influence

## FACTORS AFFECTING ABSORPTION OF SODIUM *d*L-MANDELATE FROM THE INTESTINE OF CATS

By R. C. GARRY AND I. A. SMITH, *From the Departments of Physiology and Chemistry, University College, Dundee, University of St Andrews*

(Received 20 November 1942)

Dakin [1908] suspected that certain racemic acids might exhibit in the gut preferential absorption of one or other of the optically active forms. He used various acids, one being mandelic acid. He placed a solution of the sodium salt of the racemic form in the intestine of anaesthetized animals, and then, when sufficient time had elapsed to allow partial absorption, examined the residue in the gut for optical activity. In no case did Dakin observe preferential absorption with any of the acids which he used. Cushny [1926], in his Dohme lectures, implied that these results of Dakin support the hypothesis that absorption from the intestine is a matter of simple diffusion.

Although it is now well known that absorption from the gut is not a matter of simple diffusion, it is not so clear how far stereoisomerism alone may affect the rate of absorption. In the case of xylose it was found that *d*(+)-xylose was more rapidly absorbed from the intestine of the pigeon and of the rat than was *l*(-)-xylose [Westenbrink, 1936; Davidson & Garry, 1941]. Since a solution of *d*L-mandelic acid, given intravenously to decerebrate cats, showed preferential excretion of the laevo-form in the urine at the outset [Garry & Smith, 1940], it was obviously of interest to observe once again the behaviour of sodium *d*L-mandelate during absorption from the gut.

### METHODS

Forty-six cats were used in the various experiments. They were either decerebrated or kept under full ether or chloroform anaesthesia. The abdomen was opened and food residues removed from the gut by washing through with Ringer's fluid at 38° C. The caudal end of the gut region in question was then closed and a measured volume of 2 or 3% *d*L-mandelic acid as the sodium salt run into the intestine. The mandelate solution, when run into the gut, was at body temperature. Thereafter, the cranial end of the gut segment was ligatured. The small or large intestine usually accepted about 50 ml. of solution.

After closure of the abdomen, the animal was allowed to survive for a period thought to be sufficient to allow absorption of anything from  $\frac{1}{2}$  to  $\frac{3}{4}$  of the salt in the gut lumen. At the end of this period the cat was killed, the intestine removed, and the gut contents recovered by washing through with 1 l. warm water. The mandelic acid was extracted with ether and the optical activity determined in acetone as previously described [Garry & Smith, 1940]. The extracted material, without further purification, showed, on analysis and on determination of the equivalent, very satisfactory agreement with the theoretical values for pure mandelic acid.

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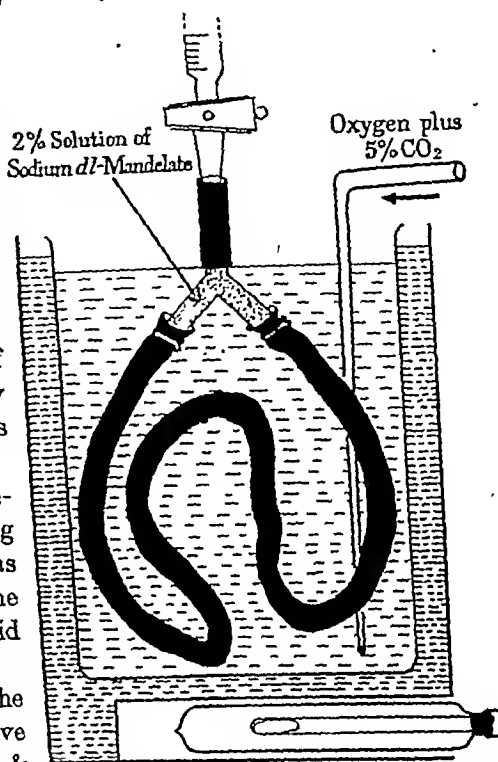


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## DISCUSSION

There are two obvious criticisms. It is possible that enzymes in the gut, and intestinal bacteria, may attack preferentially the laevo-form of mandelic acid, thus preventing its subsequent extraction with ether. Or dextro-rotatory interfering substances may accompany the mandelic acid during the process of extraction. The observations *in vitro*, and the experiments with cats killed by bleeding, where the above factors were still operative, seem to exclude such possibilities. In addition, we incubated an aqueous solution of sodium *dl*-mandelate at 38° C. with faecal matter. The acid extracted after incubation was optically inactive.

Earlier workers, in an endeavour to explain intestinal absorption in terms of simple diffusion, osmosis and filtration, made great play with the marked disappearance of solutes from the lumen of dead or dying intestine. More than 40 years ago, such endeavours were vigorously attacked by Waymouth Reid [1890, 1892*a*, 1892*b*, 1896, 1897, 1900*a*, 1900*b*], who showed that integrity of the living intestinal epithelium plays a dominant role in absorption and that factors other than simple diffusion, osmosis and filtration are at work. He even went the length of postulating the existence of 'molecule valves' in the striated border of the epithelium of the small intestine. Our present results support his views, and it would seem that, in the case of mandelic acid at least, similar 'valves' must exist in the mucus-secreting epithelium of the colon. The experiments with ether and chloroform anaesthesia suggest, too, that the processes concerned in the selective absorption of mandelic acid are very delicate. Dakin's failure to show preferential absorption of one isomeride of mandelic acid from the intestine was probably due to his use of anaesthesia. However this may be, it is obvious that the intestinal epithelium, both in small intestine and in colon, does take cognisance of the molecular architecture of mandelic acid during the course of absorption.

## SUMMARY

From the small intestine and from the colon, in decerebrate cats, there is slight but unequivocal preferential absorption of the laevo-form of mandelic acid.

Such preferential absorbing power is not shown during ether or chloroform anaesthesia of the cat, is absent from the gut kept alive *in vitro*, and does not survive in the gut of cats recently killed.

The selective absorbing power seems to be associated with the integrity of the intestinal epithelium.

Our thanks are due to the Carnegie Trust for the Universities of Scotland for a grant to cover expenses.

of ether or chloroform, the acid recovered from the gut remained optically inactive. The mandelic acid in the urine, however, still showed laevo-rotation, but not to the same extent as in the decerebrate cats (Table 1 (c)).

*Dead cats.* Immediately after the solution of sodium *dl*-mandelate was placed in the intestine, the cat was killed by bleeding from the carotids or by intravenous injection of a solution of sodium fluoride or of sodium cyanide.

Rather surprisingly, a considerable quantity of the mandelate salt disappeared from the small intestine within an hour or so. Longer duration in the gut did not substantially increase the amount disappearing from the intestine. The acid recovered from the gut lumen was, in every case, optically inactive (Table 1 (d)).

A considerable amount of mandelic acid also disappeared from the colon of dead cats, although more slowly than from the small intestine. The recovered acid was again optically inactive (Table 1 (e)).

In the dead cats marked desquamation of the intestinal mucous membrane took place while the sodium mandelate was in the gut. This did not occur in the living cats.

TABLE 1. Factors affecting absorption of sodium *dl*-mandelate from the gut. Optically pure mandelic acid has a rotation of  $[\alpha]_{D_{25}} \pm 189.9^\circ$  in acetone

	Region	Condition of cat	Duration of solution in gut min.	Percentage recovery from gut	Rotation of acid $[\alpha]_{D_{25}}$	
					From gut	From urine
(a)	Small intestine	Decerebrated	70	26	+13.5°	-73.0°
(b)	Colon	Decerebrated	607	50	+10.2°	-72.0°
(c)	Small intestine	Ether anaesthesia	60	25	Optically inactive	-25.0°
(d)	Small intestine	Killed by bleeding	240	43	Optically inactive	—
(e)	Colon	Killed by bleeding	240	74	Optically inactive	—
(f)	Small intestine	In vitro	240	44	Optically inactive	—

*The small intestine in vitro.* For several hours the gut in vitro showed active peristalsis, driving the contained mandelate solution round and round in a circle. The neuro-muscular mechanism was obviously very much alive. However, microscopic examination of the gut, and of the material within the lumen, showed that, as in the dead cats, early desquamation of the epithelium was taking place.

Mandelic acid disappeared quite rapidly from the gut lumen in vitro. Some soaked into the substance of the gut wall, from which we later recovered it by maceration and extraction, while a smaller amount went right through the thick intestinal wall into the surrounding Ringer fluid. The mandelic acid, whether recovered from the residue inside the gut, from the substance of the gut wall, or from the surrounding Ringer solution, was optically inactive (Table 1 (f)). In the experiment quoted in Table 1 (f), 13% of the acid was recovered from the solution outside, and 25% was extracted from the substance of the gut.

# RECOVERY OF FIBRE NUMBERS AND DIAMETERS IN THE REGENERATION OF PERIPHERAL NERVES

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In a normal mixed peripheral nerve it is possible to distinguish different groups of fibres, characterized by different diameters and degrees of medullation. These groups are responsible for different components in the conducted action potential of the nerve and subserve different functions [Grundfest, 1940; Zotterman, 1939]. When such a mixed nerve is interrupted all the axons and myelin in the peripheral stump degenerate, and reinnervation is accomplished by the outgrowth of axons from the central stump. Therefore, if the fibre-size groups of normal nerve are to be regarded as having any functional significance, regeneration should be directed towards the provision of an assemblage of fibres in which the numbers, diameters, medullation, and central and peripheral connexions of the fibres in each group approximate to those seen in normal nerve. In the regeneration of a particular nerve it should be possible to distinguish two processes: (1) *outgrowth*, which has as its result the arrival and connexion of fresh axons with the periphery, and (2) *reconstitution*, by which the numbers, diameters, and medullation of the fibres are restored.

Although these two processes are largely coincidental, the reconstitutive aspect of regeneration has received relatively little attention. It is well known that the first fibres laid down in regeneration are thin and non-medullated, and that they later thicken, become medullated, and acquire nodes and incisures [Hentow, 1933]. It has been stated that this process of thickening and medullation has a downward trend, spreading as a wave front along the nerve some distance behind the front of advance of axon tips [Howell & Huber, 1892; Kirk & Lewis, 1917; Sanders & Young, 1942], although in individual fibres it may proceed discontinuously [Speidel, 1932; Clark & Clark, 1938]. However, Davenport, Chor & Cleveland [1939] found that 3 months after division and suture of a nerve the ratio of the number of unmyelinated to myelinated fibres remained unchanged, and concluded that 'growth and

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*Histological methods*

At one of various times after operation each animal was killed and a number of pieces of nerve removed for fixation and staining. Pieces from the following sites were taken: (a) from the central stump 15 mm. above the crush, suture, or graft; this was regarded as being sufficiently far above the lesion to avoid gross effects of retrograde degeneration and subsequent regeneration; (b) from the peripheral stump 10 mm. below the crush, suture or graft; (c) in the case of grafts, from the middle of the graft, 10 mm. below the upper junction; (d) in the case of certain crushes extra pieces were taken at 40 and 80 mm. below the lesion. This lowest level was just above the entry of the nerve into the most proximal of the muscles supplied by it.

Each piece of nerve was attached to a piece of library card during fixation and dehydration to prevent its curling up, and care was taken to avoid stretching the pieces either before or during fixation.

Precautions were taken to ensure that all the pieces received identical treatment during fixation and staining. All the pieces from one animal were fixed in the same vessel, carried through the processes of dehydration and impregnation with paraffin together, embedded in the same block, cut together, and mounted and stained upon the same slide. The sets of pieces from different animals were treated comparably by leaving them for identical periods in the various solutions.

After numerous unsuccessful attempts the following method was found to result in minimal distortion of the myelinated fibres: fixation in Flemming's fluid followed by impregnation and embedding in 56° C. paraffin; transverse sectioning at 4-5  $\mu$ ; staining with Kulschitzky's haematoxylin and differentiating according to the method of Wolters [Romeis, 1937]. This method gave very clear preparations in which myelinated nerve fibres appeared as darkly stained rings on a light ground. Degenerated myelin was also stained and appeared as black, irregular, granular masses.

*Counting method*

Selected sections were projected on to white paper at a magnification of 450  $\times$ , this being the largest magnification at which an image of the whole section could be obtained with the objectives at our disposal, and all counts and measurements were made upon the magnified image so obtained.

In order to avoid the time-consuming labour of counting every fibre in every section in a relatively large mass of material, a sampling method was employed. A ruled ocular grid was projected at the same time as the section, so that the image on the paper appeared divided into a number of squares. The section occupied 30-50 of these squares. Counts were made of all the myelinated fibres in eight squares scattered over the section (1200-1700 fibres), and the total number of fibres in the section estimated from the number in this sample and the total number of squares occupied by the image of section.

*Measurement of fibres*

A sampling method was also used in measuring the fibres. Again, to allow a large material to be dealt with fairly rapidly, exact measurements of individual fibres were not made (except in some cases; see below, p. 492), the number of fibres falling within the limits of certain arbitrary size groups being estimated in the following manner. By means of an engraved scale a pair of dividers could be set to provide a series of templates, differing by steps of equal magnitude, each step at a magnification of 450  $\times$ , representing a step of 1.8  $\mu$  on the section. The dividers were first set so that their points were separated by one scale division (1.8  $\mu$  on the section), and all the fibres in one square of the magnified image of the section whose outside diameters were smaller than this diameter were counted and marked off. Next the separation of the divider tips was advanced to two divisions (3.6  $\mu$ ) and the process repeated; then to three divisions (5.4  $\mu$ ) and so on up the scale. When all the fibres in one square of the section had been measured in this way, those of a second square were measured, and so on until all eight squares had been dealt with. In this way, the proportionate number of fibres in successive groups at 1.8  $\mu$  intervals could be estimated relative to the total number in the sample. To make these results comparable for the different nerves, all sets of measurements were reduced to a basis of a total sample of 1000 fibres. Since the number of fibres measured in every case exceeded 1000, this procedure did not introduce an error.

myelinization proceed almost simultaneously'. Moreover, there are no observations of the extent to which the original pattern of fibre sizes within the nerve is reproduced during regeneration.

The degree to which the number of fibres in the nerve is restored during regeneration has never been thoroughly investigated. Many authors [see Aird & Naffziger, 1939] claim that after suturing the central stump of a nerve of small diameter to a peripheral stump of larger diameter, more fibres were found in the peripheral stump than in the central. However, both Greenman [1913] and Davenport, Chor & Dolkart [1937] found that after connexion of a central stump with a peripheral stump of the same diameter, fewer fibres were found in the peripheral stump as late as 6 months after operation.

In previous papers [Gutmann, 1943; Gutmann & Sanders, 1943] clear differences were described in the degree of functional recovery obtained following repair of the peroneal nerve of the rabbit by different surgical procedures. Differences were also seen in the maximum diameter attained by the fibres in the regenerating nerve trunks, at times long after functional recovery had reached its maximum degree. The present observations were made in order to define more precisely these differences in myelination, and to discover whether there was any correlation between the number and size grouping of the myelinated fibres in the nerve and the degree of recovery attained.

The peroneal nerve of the rabbit was interrupted in the thigh by (a) simple local crushing, or (b) severance. After severance it was repaired by either (a) end-to-end suture, or (b) various nerve grafts. At different times after operation pieces of nerve were taken from each animal and counts and measurements made of the myelinated nerve fibres (a) in the central stump well above the lesion, (b) at various levels below the crush, suture, or graft, and (c) within the various grafts. Comparisons of the fibre numbers and sizes in these situations were made with the results of measurements made upon normal nerves at similar levels.

## METHODS

### *Technique of operation*

The animals used were adult rabbits of both sexes. No attempt was made to use animals of a standard race, age, or body weight. At an initial operation under anaesthesia (nembutal and ether) the peroneal nerve of one side was interrupted either by (a) crushing with fine watchmaker's forceps, or (b) cutting with sharp scissors. After cutting, the nerve was repaired either by junction with cockerel plasma in the way described by Young & Medawar [1940] or by the insertion of nerve grafts of a standard length of 2 cm., fixed in place with plasma as described by Sanders & Young [1942]. These grafts were either (1) autografts, (2) fresh homografts, (3) homografts previously stored for 7 days in Ringer's solution at 2° C., or (4) alcohol-fixed grafts.

After operation the time of return of reflex spreading of the toes was followed, and subsequent improvement in the power of the reflex estimated by the 'spreading index' described by Gutmann [1943].

*Histological methods*

At one of various times after operation each animal was killed and a number of pieces of nerve removed for fixation and staining. Pieces from the following sites were taken: (a) from the central stump 15 mm. above the crush, suture, or graft; this was regarded as being sufficiently far above the lesion to avoid gross effects of retrograde degeneration and subsequent regeneration; (b) from the peripheral stump 10 mm. below the crush, suture or graft; (c) in the case of grafts, from the middle of the graft, 10 mm. below the upper junction; (d) in the case of certain crushes extra pieces were taken at 40 and 80 mm. below the lesion. This lowest level was just above the entry of the nerve into the most proximal of the muscles supplied by it.

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### Recording

From the results of these counts and measurements the following estimates were made: (1) the total number of myelinated fibres in the section; (2) the number and percentage of fibres greater than a certain arbitrary diameter ( $8\mu$ )—the reasons for the choice of this diameter are discussed below (pp. 494–5); (3) the 'average-fibre' diameter of the nerve—this quantity was obtained by multiplying the number of fibres in each size group of the sample by the diameter of the mean fibre of the group, summing the values for all the size groups, and dividing the result by the total number of fibres in the sample, and (4) the *size factor*. It will be shown that the maturation of new fibres in the peripheral stump during regeneration is accompanied by a decrease in size of the fibres in the central stump, and that the 'ontgrowth' of nerve fibres has the essential character of an outflow. The *size factor* was devised to give a measure of this outflow. The value for this factor at a particular level measures the volume occupied by myelinated nerve fibres in a unit length of nerve at this level, relative to other levels in the same nerve. It is thus a measure of both the number and the size of the fibres present. To obtain the *size factor* the total number of fibres in each size group per 1000 total fibres was multiplied by the square of the *radius* of the mean fibre of the size group, and summed throughout the nerve. This total was then, in the case of peripheral stumps, multiplied by the fraction of central stump fibres represented in the periphery. Data were recorded in the form of (a) tables, and (b) frequency block diagrams, in which the number of fibres in each size group was plotted against fibre diameter (e.g. see Fig. 1).

### Sources of error

**Fixation.** All measurements were made upon fixed and stained nerve fibres. Comparative measurements of fresh and osmium tetroxide-fixed nerve fibres have been made by a number of authors [Sherrington, 1894; Donaldson & Hoke, 1905; Duncan, 1934], and the general conclusion is that osmium-tetroxide fixation followed by paraffin embedding does not cause more than 10% shrinkage in the diameter of myelinated nerve fibres. Hursh [1939], who employed the Kulschitzky method for staining myelinated fibres, found shrinkage of the same order. Thus the absolute fibre diameters recorded may be slightly less than those which would be obtained from fresh nerve fibres. However, the comparative measurements obtained from different nerves by this method are unaffected by this source of error, since all the pieces were treated identically during fixation and staining.

**Variations in magnification.** This source of error was reduced to a minimum by checking the magnification with a micrometer slide immediately before each nerve was measured.

**Errors of measurement.** Counting the fibres within the various size groups at a magnification of  $450\times$  probably involves error, especially with the smallest groups. Ten successive counts of the fibres of the  $0\text{--}1.8\mu$  size group in the same square of a section of normal nerve gave numbers of 28, 26, 28, 27, 29, 28, 28, 26, 28, 32. These errors are not likely to be of sufficient magnitude to disturb the results.

**Errors of sampling.** In two normal nerves the magnitude of this error was estimated by first calculating the total number of fibres in the section by the sampling method described, and then by making a complete count of all the fibres in the section. In the first nerve, the sampling method gave a total number of fibres of 7120, the total count 7009. In the second, calculation gave a total fibre number of 6204, while a direct count gave 6496. In the first of these estimations the error is 1.6%, in the second 4.5%. It is probably safe to assume that this method does not involve any very large error.

**Irregularity in the contour of individual fibres.** Although distortion in the outline of the fibres due to fixation and staining is minimal, not all the fibres in any one section appear as perfect circles. A section may contain both oval and crenated fibres, and where these were found actual measurements were made of their diameters. In the case of oval fibres two measurements were made of the diameter of each fibre, one along the long axis, and one at right angles to it. In the case of the crenated fibres two measurements were made of the greatest diameter of each fibre. The mean of the two measurements was taken as the actual diameter of both oval and crenated fibres. Duncan [1934] states of such mean measurements that 'the results are not more than



10% less than that of a circular figure'. This 10% was not taken into account unless the mean diameter was found to be on the border-line between two size classes, when the fibre was included in the higher class.

*Degenerated fibres.* Degenerated myelin normally appeared in the sections as irregular black granular masses, and the degenerated fibres could easily be distinguished from normal fibres, which stained as firmly outlined black rings. Only obvious rings were counted as nerve fibres. In very early stages, however, transversely cut myelin 'ellipsoids' may have the appearance of normal fibres. Cajal [1928] states 'from the 10th day onward nearly all the large ellipsoids have become very numerous fatty droplets'. Since the earliest stage studied was 50 days after operation, it is certain that very few ellipsoids would be present, and even if mistaken for normal fibres, the error introduced would be very small.

*Variations in diameter along the course of individual fibres.* The significance of this factor has been discussed by Duncan [1934], who made actual measurements of the variations in diameter along the course of nerve fibres in teased preparations and found 'variations in diameter amounting to 100%, but in each case over one half of the measurements fall within  $1\mu$  of the mean'. Apart from the very great change at the nodes, the principal constriction of the myelin occurs opposite the Schwann nuclei [see Holmes & Young, 1942 for figure]. As it is probable that considerable variations in diameter occur along the course of living nerve fibres, no attempt has been made to eliminate this factor. However, it has been asserted that nerve fibres normally undergo a conical diminution in diameter during their course down a peripheral nerve (Björkman & Wohlfart, 1936). To eliminate this factor, sections were taken from comparable levels in the nerve as far as was possible, and all lesions were made at a standard distance (80 mm.) above the first muscle branch given off from the nerve in its downward course.

*Age and size of animals.* No attempt was made to control this factor. All the animals were adults, but varied considerably in size and body weight. Consequently a considerable variability in fibre number was found in the central stumps and normal nerves examined, but in every case where an abnormally large fibre number was found, the specimen had been taken from a large animal and vice versa. Moreover, since sections were taken from the central stump in every case, as well as from peripheral levels, the fibre numbers peripheral to the lesion could be directly compared with the number central to it and the effects of this variability eliminated.

## RESULTS

### *The fibre content of normal nerves*

Preparations were made of the peroneal nerves of five unoperated rabbits. Table 1 shows the estimated total number of myelinated nerve fibres present in each case. In one case three estimations of the total number of fibres in the nerve were made at three levels in the nerve corresponding to the levels at which sections were taken in some of the experimental animals (see above p. 491). From Table 1 it will be seen that the total number of fibres in the

TABLE 1. Numbers and sizes of fibres in normal rabbit peroneal nerves

Nerve	Total no. of fibres	No. of fibres larger than $8\mu$	Percentage of fibres larger than $8\mu$	'Average-fibre diameter' $\mu$	'Size factor'
1 (a)	6150	1224	19.9	5.56	12,009
(b)	6140	1013	16.5	5.27	11,932
(c)	6204	1061	17.1	5.36	12,203
2	6725	1178	17.5	5.28	11,846
3	7120	1213	17.3	5.41	12,043
4	8213	1462	17.8	5.32	12,250
5	9117	1543	16.9	5.40	12,603
Mean			17.6		

nerve varied roughly between 6000 and 9000, while there was no significant difference in the number of fibres at the three levels in the nerve.

Fig. 1 is a chart of the relative numbers of fibres in the various size groups per 1000 total fibres in sections of a normal nerve taken at the three levels described. At all three levels there is a range of fibre sizes present, the largest group having a mean diameter of  $18.9\mu$ . The method of size grouping used in obtaining these results obscures the fact seen by Duncan [1934] that there is a critical diameter (about  $1\mu$ ) below which no fibres are myelinated, and above which all fibres are myelinated. Actually the fibres in the smallest group ( $0-1.8\mu$ ) were all near the upper limit of the group. No myelinated fibres were seen smaller than about  $1-1.5\mu$  in diameter; and hence the  $0-1.8\mu$  fibre group really represents a  $1-1.8\mu$  fibre group.

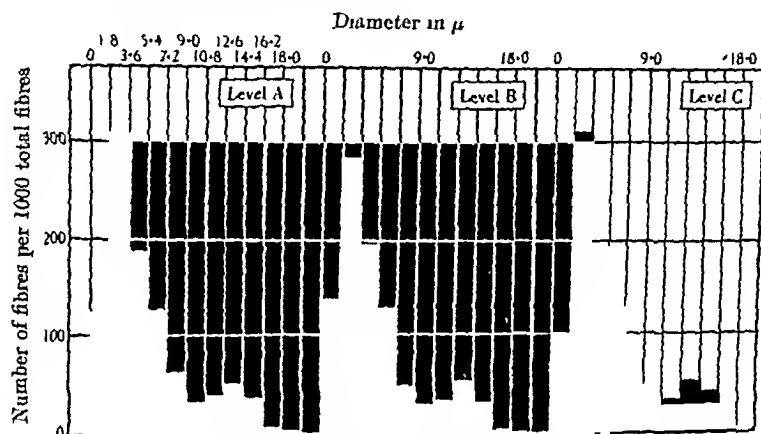


Fig. 1. Histograms to show the fibre-size spectrum at three levels in a normal nerve, corresponding with the three levels taken in the case of nerve grafts (see text, p. 491).

The histograms of the distribution of fibre size in the nerve given in Fig. 1 show that the majority of the fibres are small. Approximately 82.4% of the fibres have a diameter less than  $8\mu$ . Thus the histograms show a large peak in the small-fibre zone, this peak falling within the  $1.8-3.6\mu$  size group. The remaining 17.6% of fibres cover the range  $8-19.8\mu$ , and their distribution shows a small peak in the  $12.6-14.4\mu$  group. In fact the fibre-size histogram can be conceived as consisting of two distinct populations of measurements, one large population with a mean at  $1.8-3.6\mu$ , and a second smaller one with a mean at  $12.6-14.4\mu$ . The upper limit of the large population probably overlaps to some extent with the lower limit of the small population, the intersection taking place somewhere in the region of the  $7.2-9\mu$  size group. The site of this intersection has been taken note of in calculating the figures given in the columns of Table 1 and succeeding tables which show the number and percentage of fibres 'larger than  $8\mu$ '.  $8\mu$  is approximately the mean fibre

diameter of the fibres in the  $7.2-9\mu$  size group, and it has been arbitrarily assumed that all fibres larger than  $8\mu$  fall within the limits of the small population of large fibres shown in the histograms of normal nerve (see Fig. 1). To say that a stump contains fibres of a 'large fibre group' or 'larger than  $8\mu$ ' therefore means that there are fibres present whose outside diameters are too large for their inclusion in the  $7.2-9\mu$  or any smaller size group. It has been stated that mammalian A fibres range in size from 20 to  $1\mu$  [Gasser & Grundfest, 1939; Hursh, 1939], so the second small population must contain the largest of these, probably the  $\alpha$  fibres. The larger population of small fibres will contain the rest of the A fibres and also the B-fibre group, whose fibres do not exceed  $3\mu$  in diameter [Grundfest, 1939]. No doubt subgroups would be revealed by more accurate measurement, but these two main groups were seen in all the normal nerves examined, and were present at all levels in the nerve.

On the basis of the charts shown in Fig. 1 estimates were made of the number and percentage of fibres in the nerves which fall within the large-fibre distribution. As the extent of overlapping between the two distributions is not known, the lower limit of the large-fibre group was fixed arbitrarily at  $8\mu$ , and the total number and percentage of fibres in the nerve larger than this diameter estimated. In addition the 'average-fibre diameter' of the nerve was calculated in the way already described. The values of these quantities for the various normal nerves are given in Table 1. The percentage of large fibres varied between 16.5 and 19.9 and the average fibre diameter between  $5.27$  and  $5.56\mu$ . Table 1 shows no steady decrease in fibre diameter as one approaches more peripheral levels. What differences there are are attributable to the errors of the method. If such a decrement exists, it is too slight to be detected by the relatively crude methods used here.

#### *The fibre content of the nerve after crushing*

Myelinated fibres were counted and measured 50, 60, 70, 90, 100, 130, 200, 250 and 300 days after operation in the central and peripheral stumps of nerves which had been interrupted by sharp localized crushing.

*Central stump.* Table 2 shows the number of fibres in the central stump 15 mm. proximal to the site of crushing at various times after operation. The nerve contained about the same number of myelinated fibres, whatever the period intervening between the operation and the taking of specimens. Apart from three values recorded from very large animals, these numbers fall within the limits of the numbers seen in normal nerves (see Table 1). Indeed, comparison of Table 2 with later tables (Tables 3, 5 and 6) shows that, whatever the lesion, injury and repair of the peroneal nerve of the rabbit do not cause any appreciable change in the number of fibres in the central stump 15 mm. above the lesion.

TABLE 2. Numbers and sizes of fibres 15 mm. central to the lesion at different times after crushing the nerve

Animal	No. of days	Total no. of fibres	Total of fibres larger than $8\mu$	Percentage of fibres larger than $8\mu$	'Average-fibre' diameter $\mu$	'Size factor'
778	50	9630	—	—	—	—
727	60	6864	1304	19.0	5.02	10,589
725	70	8798	1206	16.9	5.00	10,551
714	90	6216	1025	16.5	4.83	9,822
707	100	9858	1251	12.7	4.47	8,572
702	130	9812	1354	13.8	4.61	8,631
703	200	8844	1689	19.1	5.30	11,633
706	200	8267	1676	20.2	5.40	11,885
715	250	7347	1499	20.4	5.55	12,175
704	300	5943	1242	20.9	5.71	12,932

Fig. 2a shows a chart of the relative number of fibres in the various size groups in a central stump at this level 130 days after the interruption of the nerve by localized crushing. As in normal nerve the majority of the fibres

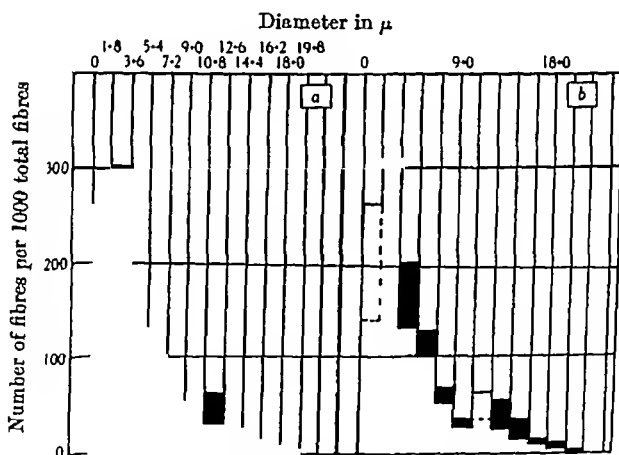


Fig. 2. Histograms of the fibre-size spectrum (a) 15 mm. above the lesion in a nerve crushed 130 days previously, (b) the same central stump histograms as (a) (unshaded) superimposed upon that for a normal nerve at the same level (shaded black).

are less than  $8\mu$  in diameter. Moreover, the histogram still preserves its general shape, so that the peaks indicating the two separate groups of large and small fibres are discernible. The peak in the distribution of the large-fibre group occurs, however, at  $10.8-12.6\mu$  instead of at  $12.6-14.4\mu$  as in normal nerve. The peak in the small-fibre group still occurs at  $1.8-3.6\mu$ , but there are more fibres in the  $0-1.8\mu$  group than in normal nerve. Indeed, there seems to have been a general shift towards a smaller size of fibre. Fig. 2b shows this effect more clearly. In this diagram the distribution chart for this central stump is shown superimposed upon that of a normal nerve at the same level, and

indicates that a general diminution in fibre size has indeed taken place throughout the range, although the general pattern of fibre sizes is preserved.

This effect is also shown by the average fibre sizes, the percentages of large fibres, and the 'size factors' given in Table 2 for all stumps younger than 130 days after operation. In these cases the average fibre size varied between  $4.47$  and  $5.02\mu$  (cf.  $5.27$  and  $5.56\mu$ , the limits of variation in normal nerve). The percentages of large fibres varied between  $12.7$  and  $19\%$  (cf.  $16.5$  and  $20\%$  in normal nerve), and the 'size factor' between  $8572$  and  $10,589$  (cf.  $11,846$  and  $12,603$  in normal nerve).

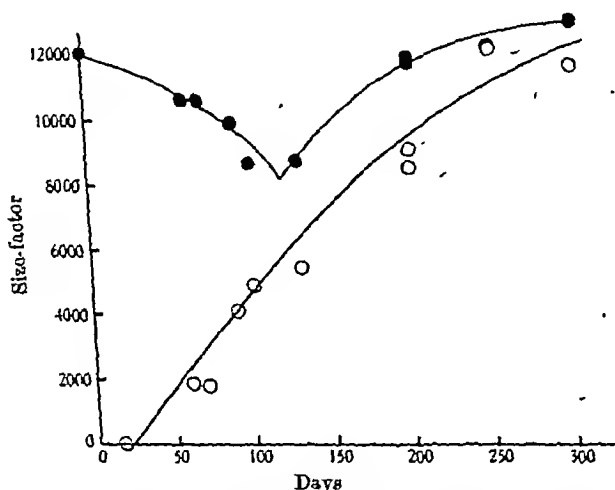


Fig. 3. Graphs to show the different values assumed by the 'size factor' (see text) in both central and peripheral stumps at different times after interruption of the nerve by crushing. Black circles: central stump values. White circles: peripheral stump values. The lines are drawn by eye.

In the 200-, 250- and 300-day stumps no such reduction in fibre diameter was seen. The average fibre diameter varied between  $5.30$  and  $5.71\mu$ , the percentage of large fibres between  $19.1$  and  $20.9$ , and the 'size factor' between  $11,633$  and  $12,932$ . Thus the fibres in the central stump above a crush seem to undergo a general diminution in diameter for at least 130 days, though at all later stages the fibres are restored to their normal diameters. Fig. 3, in which the size factors for both central and peripheral stumps are plotted against time, shows clearly the diminution and eventual restoration of the fibre sizes in the central stump. Similar decreases in diameter occurred in the central stumps after severance of the nerve and its repair by suture or grafting (see Tables 5 and 6).

A similar diminution in diameter of the fibres above an interruption was seen by Greenman [1913]. The outgrowth of a nerve fibre is generally accepted

as proceeding through the 'amoeboid' activity of its tip, travelling down a peripheral pathway spinning out a fibre behind it [Harrison, 1910; Weiss, 1941]. However, it is not known whether the subsequent increase in diameter of a fibre takes place by the synthesis of new protoplasm within the outgrowing fibre, or whether it is brought about by an outflow of axoplasm into the new stretch of fibre from the cell body and the stretch of fibre proximal to the lesion. Young [1935] showed that severance of the giant axons of *Sepia* was followed by an outflow of axoplasm from the cut end of the fibre, and Young & Seddon [personal communication] have shown that the early 'outgrowth' of axons across crushes and suture lines has the essential character of an outflow. In this case it would be only logical to expect a decrease in diameter

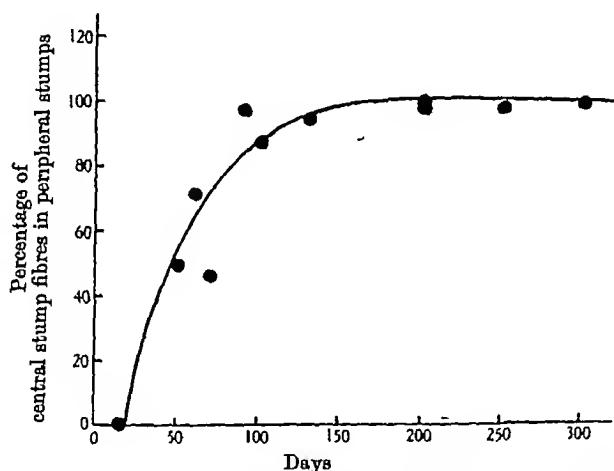


Fig. 4. Total numbers of myelinated fibres in the peripheral stump (expressed as percentages of the total number of fibres in the corresponding central stumps) at different times after interruption of the nerve by crushing.

in the central portion to accompany the advance and maturation of new fibres within the peripheral stump. Thus it appears probable that the advance and maturation of new fibres within a regenerating nerve does not represent a true 'growth' but is essentially an outflow from the cell body which itself may undergo a decrease in diameter.

This outflow, however, goes on for only about 130 days. In the 200-, 250- and 300-day stages the fibres are restored to their normal diameters. The fibres in the central stump first of all flow out, which causes them to decrease in diameter, and then undergo some form of 'growth' which enables them to increase in diameter once more. Whether this is a true 'growth' or is the result of further outflow from the cell body and the more central portions of the fibre is not known. It is suggestive that the point of inflexion of the curve (see Fig. 3) where the fibres reach their lowest diameter and outflow begins to be

replaced by 'growth', is also the time at which both the numbers and the diameters of the fibres in the periphery begin to approach those of the fibres in the central stump (see below, and Figs. 3, 4).

*Peripheral stumps.* Table 3 shows the number of myelinated fibres in the peripheral stump below crushes at various times after operation. To make the figures for the different animals comparable, the number of fibres in the various peripheral stumps have also been expressed as percentages of the number of fibres in the central stumps above them (see Table 3, col. 4). These percentages are shown plotted against time in Fig. 4. It will be seen that the percentage of central stump fibres represented in the peripheral stump gets progressively larger, and at about 150 days after operation becomes equal to 100%. In Fig. 4 the starting point of the curve, the point at 0%, is derived from the data of Young [1942]. Myelinated fibres appear at this level (10 mm. below the lesion) at about 15 days after operation. The number of fibres in the peripheral stump reaches a maximum at about the time the diameters of the fibres of the central stump reach a minimum, and thereafter begin to increase in size.

TABLE 3. Numbers and sizes of fibres 10 mm. peripheral to the lesion at different times after crushing the nerve. C.S. = central stump

Animal	No. of days	Total no. of fibres	Percent- age of fibres in C.S.	No. of fibres larger than $8\mu$	Percent- age of fibres larger than $8\mu$	Percent- age* of large fibres in C.S.	'Average- fibre diameter $\mu$	'Size factor'
778	50	4826	50	—	—	—	—	—
727	60	4958	72	0	0	0	2.84	1,946
725	70	4029	46	34	0.8	3	3.32	1,730
714	90	6128	98	220	3.6	21	3.46	4,724
707	100	9557	87	522	6.1	32	3.85	4,893
702	130	9288	95	697	7.5	51	3.93	5,494
703	200	8792	99	1301	14.8	77	4.98	9,186
706	200	8353	101	1119	13.4	67	4.67	8,366
715	250	7192	98	1410	19.6	90	5.74	12,030
704	300	5956	100	1131	19.0	91	5.46	12,056

\* The figures in this column in Tables 3-6 are derived from

$$\frac{\text{no. of large fibres in the peripheral stump}}{\text{no. of large fibres in the central stump}} \times 100,$$

and thus represent the fraction of large fibres in the central stump which correspond to large fibres in the periphery.

Table 3 also shows the numbers and percentages of large fibres (greater than  $8\mu$ ) in these animals, and shows that the myelinated fibres increase in diameter. At 60 days there were no fibres greater than  $8\mu$ , while at 250 and 300 days there were 1410 and 1131 such fibres, total percentages of 19.6 and 19.0, comparable with the figures for normal nerve (see Table 1). To make the various animals comparable the numbers of large fibres were also expressed as fractions of the number of large fibres present in the corresponding central

stumps (see Table 2). These are shown plotted against time in Fig. 5. It will be seen that there was a progressive increase in the number of large fibres in the peripheral stumps, and that these reached comparable numbers with the fibres of the central stumps only after 250 and 300 days.

The average fibre diameters and 'size factors' also showed comparable increases. At 250 days both showed values comparable with those for normal nerves. Fig. 3 shows the 'size factors' for the peripheral stump plotted against time on the same scale as those of the corresponding central stumps. While the fibres of the central stump undergo a diminution followed by an increase in diameter, those of the peripheral stump increase progressively. After about 150–200 days they have reached diameters comparable with those of the central

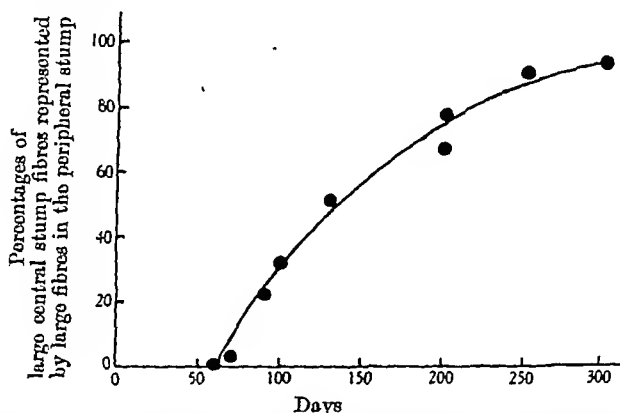


Fig. 5. Numbers of myelinated fibres in the peripheral stump larger than  $8\mu$  in diameter (expressed as percentages of the number of fibres in the corresponding central stump larger than  $8\mu$ ) at different times after interruption of the nerve by crushing.

stump, and thereafter both increase in diameter together, both reaching similar values to those of normal nerves at about 250 days after operation.

All the above figures show that from 60 days onwards there is an increase both in the number and in the diameter of myelinated fibres, presumably both by the medullation of previously non-medullated fibres, and by increase in diameter of previously medullated fibres. By 130–150 days after operation, the number of medullated fibres equals that of the central stump (see Fig. 4) and thereafter does not increase greatly. Fibres go on increasing in diameter, however, until at 250 days the nerve has returned to normal.

These data are borne out by the histograms of fibre distribution of these cases given in Fig. 6. Until 130 days there is no sign of a separate peak in the large-fibre zone, although from 70 days onwards fibres greater than  $8\mu$  are present. After the small-fibre peak in the  $1.8$ – $3.6\mu$  zone the number of fibres in successive size classes progressively declines. At 130 days a small secondary peak appears at  $5.4$ – $7.2\mu$ , but it is impossible to be certain whether or not this



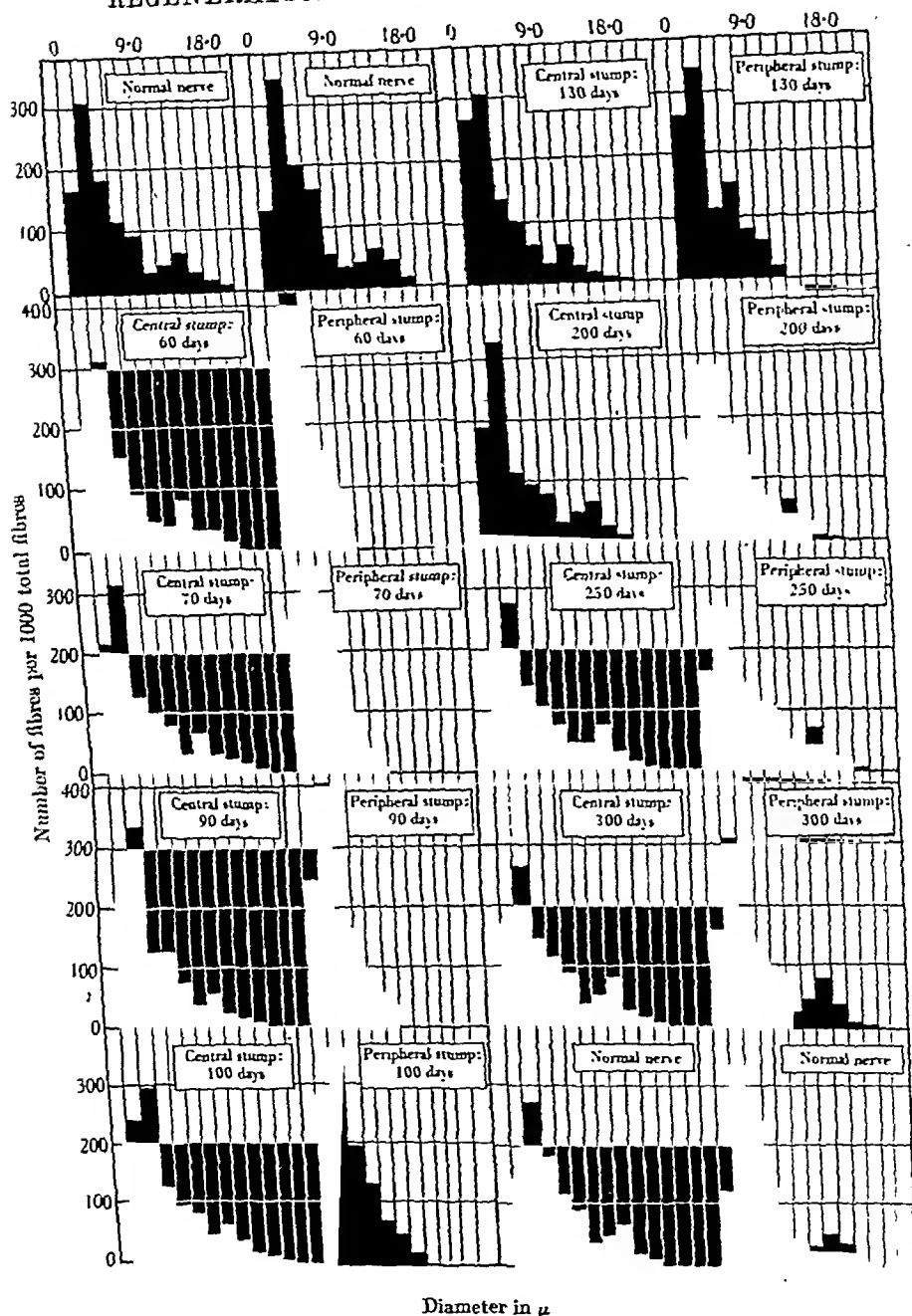


Fig. 6. Histograms of the fibre-size spectrum 15 mm. above and 10 mm. below the lesion at different times after crushing the nerve, to show how the nerve is eventually completely reconstituted. To this end the histograms for normal nerve at the corresponding levels are also given, both at the start and finish of the series.

represents the beginning of a true large-fibre peak or is fortuitous. However, no such secondary peak was ever found in this region in any other animal. At 200 days there is a well-defined peak, but it is in the  $10.8\text{--}12.6\mu$  size group. At 250 and 300 days there is a peak in the  $12.6\text{--}14.4\mu$  size group, and the frequency diagram of the stump is indistinguishable from that of normal nerve. Therefore, 250 days after interruption of the nerve by simple localized crushing, both the numbers and the pattern of fibre sizes within the peroneal nerve of the rabbit 10 mm. below the lesion are completely restored.

*Other levels in the nerve.* Measurements were also made of the number and sizes of the fibres at two further levels in the peripheral stump 60, 130, 250, and 300 days after crushing the nerve. These levels were at 40 and 80 mm. below the lesion. The results of these measurements are given in Table 4.

TABLE 4. Numbers and sizes of fibres 10, 40, and 80 mm. peripheral to the lesion at different times after crushing the nerve. C.S.=central stump

Animal	No. of days	Level in mm.	Total no. of fibres	Percent- age of fibres in C.S.	No. of fibres larger than $8\mu$	Percent- age of fibres larger than $8\mu$	Percent- age of C.S. large fibres	'Average- fibre' diameter $\mu$	'Size factor'
727	60	10	4958	72	0	0	0	2.84	1,946
		40	4703	69	0	0	0	2.64	1,673
		80	3698	54	0	0	0	2.05	845
702	130	10	9288	95	697	7.5	51	3.93	5,494
		40	8758	89	405	4.9	36	3.64	4,385
		80	7563	77	174	2.3	17	3.64	3,545
715	250	10	7192	98	1410	19.6	94	5.74	12,030
		40	7164	98	1361	19.0	91	5.35	11,157
		80	7211	98	1031	14.3	70	4.97	9,523
704	300	10	5956	100	1131	19.0	91	5.96	12,056
		40	5915	100	1153	19.5	93	5.45	11,519
		80	5926	100	1144	19.3	92	5.45	11,970

From Table 4 it will be seen that at 60 days there is a progressive decline in the total number of medullated fibres and the average fibre diameter as one passes from the lesion down the nerve. At 130 days there are more large fibres present, but the decline in fibre diameter and number on passing peripherally still persists. At 250 days the total number of fibres has been restored throughout the nerve, but at the most peripheral level there are fewer large fibres than nearer to the lesion, where the fibre-size spectrum has been restored (see p. 500). Not until 300 days after operation is the nerve entirely normal throughout its length as regards fibre size and number.

Crush injuries can, therefore, be followed by complete reconstitution of the nerve, although this state of normality is not achieved throughout the nerve for about 300 days after injury. In the first 150 days, when the small myelinated fibres in the periphery are increasing rapidly in diameter, the drain on the fibres in the central stump causes them to undergo a diminution in diameter, which is later made up. The final reconstitution of the nerve includes the

restitution of the fibre distribution histogram, which, 10 mm. below the lesion in a nerve 250 or more days after operation, is indistinguishable from that of normal nerve, including a separate population of large fibres, whose mean diameter lies at  $12.6\text{--}14.4\mu$ .

Tested by the return of reflex spreading of the toes, functional recovery began at about 42 days after crushing, and 1 week later the toes could be spread out with the same amplitude as on the normal side. Thus at 60 days, when the 'average-fibre' diameter in the peripheral stump was only  $2.84\mu$  (compared with  $5.02\mu$  in the central stump, see Table 2) and only 54% of the central-stump fibres had become medullated at the level of the muscle, the animal was able, through such a nerve, to bring about a co-ordinated contraction of the peroneal musculature indistinguishable in power from the normal movement. Presumably, since conduction rate depends upon fibre diameter, the response is more sluggish on the operated side. The nerve goes on recovering, however, long after functional recovery has reached a maximum, and is not completely reconstituted till at least 200 days after full functional recovery.

#### *Fibre content after severance and suture*

Myelinated nerve fibres were counted and measured above and below the lesion 100, 200 and 364 days after the nerve had been cut and immediately repaired by junction with plasma. The results of these measurements are given in Table 5.

TABLE 5. Numbers and sizes of fibres (a) 15 mm. above, and (b) 10 mm. below the lesion at different times after severance and repair of the nerve by end-to-end suture.  
C.S. = central stump

Animal	No. of days	Stump	Total no. of fibres	Percent- age of C.S. fibres	No. of fibres larger than $8\mu$	Percent- age of fibres larger than $8\mu$	Percent- age of large fibres in C.S.	'Average-fibre' diameter $\mu$	'Size factor'
708	100	Central	10,346	—	1257	12.2	—	4.71	8646
		Peripheral	6,768	85	114	1.7	9.1	3.48	2795
671	200	Central	5,741	—	890	15.5	—	4.88	8784
		Peripheral	4,989	87	190	8.8	21.3	3.61	3756
753	200	Central	7,580	—	1126	14.9	—	4.58	8914
		Peripheral	5,674	75	301	5.3	26.7	3.59	3848
670	200	Central	6,905	—	773	11.2	—	4.54	8277
		Peripheral	4,707	68	71	1.5	9.2	3.16	2413
752	200	Central	9,356	—	1506	16.1	—	4.85	9788
		Peripheral	6,369	68	331	5.2	22.0	3.52	3103
569	364	Central	9,813	—	1619	16.5	—	4.85	9634
		Peripheral	6,539	66	549	8.4	34.0	3.88	3598
569	364	Central	9,026	—	857	9.5	—	4.21	6995
		Peripheral	7,140	79	300	4.2	35.0	3.69	5148

*Central stumps.* 15 mm. above the site of suture the myelinated fibres of sutured nerves were found to be reduced in diameter, even as late as 364 days

after suture. One central stump at 364 days was found to have an average fibre diameter of  $4.21\mu$ , far less than the lowest diameter achieved after crushing, while others, at 100, 200 and 364 days after suture, had 'average-fibre' diameters of  $4.71$ ,  $4.54$ ,  $4.88$  and  $4.85\mu$ . The size factors, and the number and percentage of large fibres were also reduced as compared with normal nerve (compare Tables 1 and 5). At 100 days the central stump contained 12.2% of large fibres (cf. a mean percentage of 17.6 in normal nerve). Four central stumps at 200 days contained 11.2, 14.9, 15.5 and 16.1% of large fibres, while in two cases at 364 days there were respectively 9.5 and 16.5% of the large fibres in the central stump. As late as 364 days after suture, therefore, the fibres of the central stump are reduced in diameter. There was no sign of the increase in diameter which followed the initial diminution seen after interruption of the nerve by crushing.

*Peripheral stump.* Table 5 also shows the results of counting and measuring the fibres in the peripheral stump 10 mm. distal to these sutures. At 100 days after operation the peripheral stump contained 6768 fibres, 65% of the number in the corresponding central stump. The numbers and percentages of large fibres, and the average fibre size, were strikingly smaller than in the central stump.

At 200 days the fibres in the nerves of four animals were counted and measured, and Table 5 shows that the results were very variable. The percentage of central-stump fibres, represented in the peripheral stump, varied from 68 to 87. The percentage of large fibres varied from 1.5 to 8.8 and the average fibre diameter from  $3.16$  to  $3.61\mu$ , figures which are strikingly less than those found for the corresponding central stumps. Especially, there were many fewer large fibres in the periphery.

The variability in these results at 200 days can be correlated to a certain extent with variations in closeness of apposition of the stumps at the junction. As Gutmann, Guttmann, Medawar & Young [1942] have pointed out, it is almost impossible to make standard unions of cut nerves, however careful the technique employed. In Fig. 7 the diagrams, based on projections of longitudinal sections, show that rabbit 671, in which the greatest percentage of fibres and of fibres larger than  $8\mu$  were seen in the peripheral stump at 200 days, and in which was found the greatest average fibre diameter at this stage, showed the best apposition of the stumps. At one side of the nerve the apposition was ideally close, fibres running straight through from the central stump to the periphery with a minimum of criss-crossing. At the other side, apposition had not been so close with the result that a lateral neuroma and 'glioma' had been formed, fibres crossing the gap between the stumps, but undergoing much criss-crossing, and many of them becoming deviated. The other three 200-day animals all showed some separation of the stumps at the suture line. Rabbit 753, which contained more and larger fibres than rabbit 670,

showed rather less separation. Animal 752 was, however, anomalous. It showed by far the greatest separation of the stumps and only 68% of the fibres of the central stump had got into the periphery, but the percentage and number of large fibres and the average fibre size was greater than in animal 670, where the separation was much less. 752 was, however, a much younger animal than the others, and study of the sections showed that there had been a much better attempt at reorganizing a new nerve in the gap between the stumps. The fibres showed a great deal of criss-crossing, but considerable fasciculation had occurred, so that the strand connecting the stumps contained interlocking bundles of fibres rather than a confused tangle of single fibres as seen in rabbit 670.

At 364 days also, one of the junctions showed much closer apposition and contained more large fibres, and had a greater average fibre diameter than the other.

The most striking fact about these sutured nerves was that none of them, even as late as 364 days after operation, showed any sign of a large-fibre peak in the fibre-distribution histogram. After an initial peak at  $1.8-3.6\mu$ , which represents the peak of the small fibre distribution of normal nerve (see Figs. 8, 9) the number of fibres in each succeeding size class declines steadily, the largest fibre class represented at 200 days being  $16.2-18\mu$ . At 364 days the largest fibre class represented is  $18-19.8\mu$ . Therefore at 364 days after an end-to-end suture, a very small number of fibres has reached a diameter equal to that of the largest fibres of the central stump, and there is no sign of the separate large-fibre group which is so constant a feature of both central stumps and normal nerves.

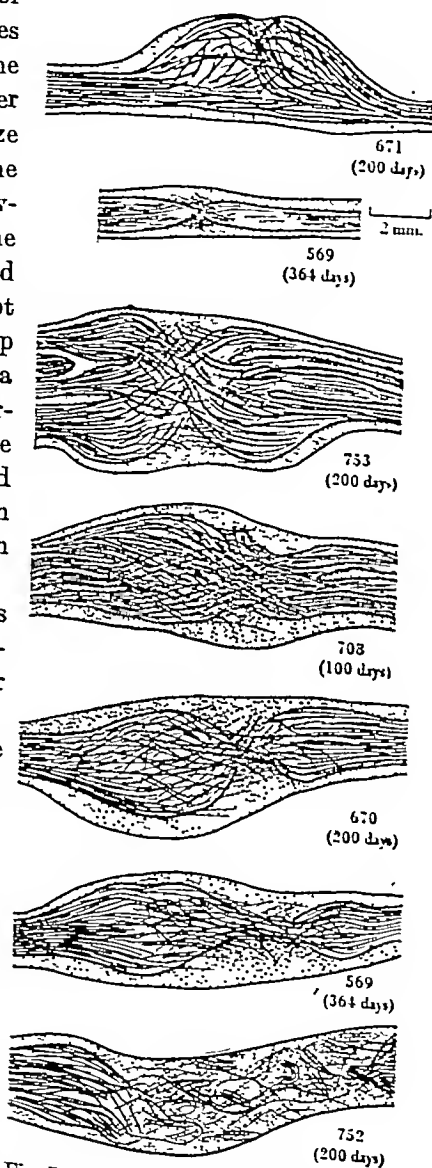


Fig. 7. Diagrams, based upon tracings of single longitudinal sections, to show the form of the stumps at the suture-line in different animals after the nerve has been cut and joined with plasma.

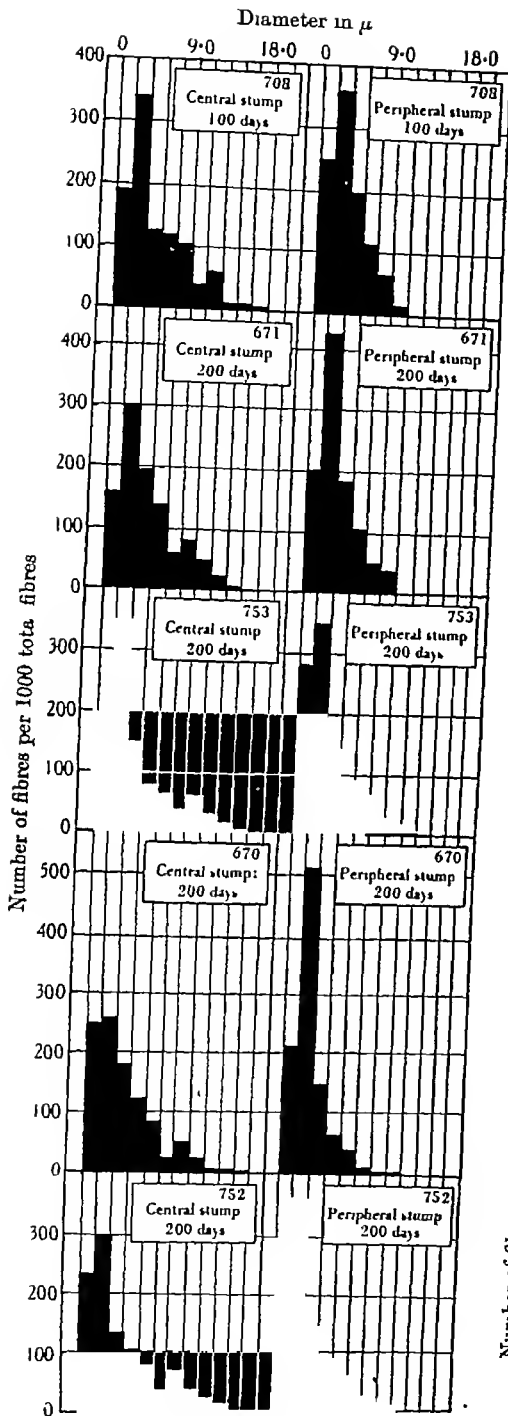


Fig. 8.

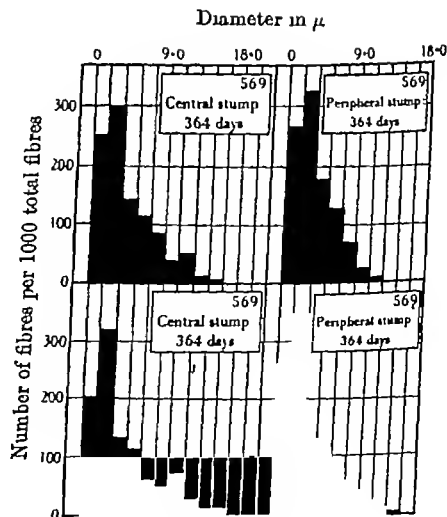


Fig. 9

Fig. 8. Histograms of the fibre-size spectrum 15 mm above and 10 mm. below a plasma suture made either 100 or 200 days previously.

Fig. 9. Histograms of the fibre-size spectrum 15 mm. above and 10 mm. below the lesion in two cases of a plasma suture made 364 days previously.

As late as 364 days after severance and suture of a nerve, then, the nerve is not completely reconstituted as far as both number and pattern of fibre sizes is concerned. Table 5 shows, however, that the 364-day stage represents a definite advance on the 200-day stage. There are more large fibres at 364 days, and the average fibre diameter is greater. Thus it is possible that reconstitution of the nerve can be still proceeding as late as 364 days after suture.

Motor recovery after end-to-end suture of the nerve 80 mm. from the muscle begins in the rabbit between 56 and 85 days after operation [see Gutmann & Sanders, 1943] and has reached its maximum within 30 days of its onset. Thus at 200 or 364 days functional recovery has been at a maximum for between 85 and 239 days. However, the maximum degree of motor recovery after end-to-end suture as measured by the amplitude of toe spreading falls definitely short of normality [see Gutmann & Sanders, 1943]. This may be correlated with the failure of the nerve to undergo proper reconstitution after end-to-end suture. Comparison of the fibre sizes after end-to-end suture with those after a crush at 130 days when spreading had been at a normal amplitude for at least 70 days, shows that although the average peripheral fibre diameter after crushing was slightly larger than after suture, the difference was hardly large enough to be significant, while at 60 days after a crush, spreading being then already normal, the fibres were smaller than those found 200 days after the worst suture. Moreover, no definite evidence of the presence of a large fibre group was forthcoming for any of these animals. The percentages of central-stump fibres found in the peripheral stumps were, however, on the average greater after crush than after suture. But these small differences in fibre numbers are not sufficient to account for the considerable discrepancy in the final quality of functional recovery attained. The only other factor which affects sutures but not crushes is that of shunting of fibres. When the nerve is crushed by a single firm localized crush, the fibres are interrupted but left opposite to their own 'Schwann tubes'. The outflowing fibre can thus pass down the peripheral stump to its original destination. In a suture, however, this is not the case. A particular fibre can easily enter one of many tubes, and the considerable criss-crossing at the suture line shows that fibres do indeed enter tubes which are not directly opposite to them. Both motor and sensory fibres may be functionally 'lost' by accidentally entering into one another's tubes. Moreover, if the size of the peripheral tube has an influence on the final diameter attained by a regenerating fibre, as the work of Holmes & Young [1942] suggests, this factor may also be responsible for the apparent failure of the peripheral stumps of nerves joined with plasma to undergo complete reconstitution. Touch, pain, and sympathetic fibres are smaller than motor and proprioceptive fibres, and if the latter become misdirected into the former's tubes, they may find difficulty in reaching their proper diameters.

*Fibre content after grafting*

Myelinated nerve fibres were also counted and measured in four cases in which the nerve had been repaired by means of a nerve graft. The grafts used were: (a) an autograft, (b) a fresh homograft, (c) a graft stored for 7 days in Ringer's solution at 2° C. prior to insertion, and (d) an alcohol-fixed graft. In each case, pieces were taken 200 days after operation: (1) from the central stump, (2) from the graft, (3) from the peripheral stump. The results of the measurements are given in Table 6.

TABLE 6. Numbers and sizes of fibres (a) in central stump 15 mm. above upper junction, (b) in graft 10 mm. below upper junction, (c) in peripheral stump 10 mm. below lower junction 200 days after the severance and repair of the nerve by a variety of 2 cm. nerve grafts. C.S. = central stump

Animal	Kind of graft	Stump	Total no. of fibres	Percent- age of of C.S. fibres	No. of fibres larger than 8 $\mu$	Percent- age of fibres larger than 8 $\mu$	Percent- age of large fibres in C.S.	'Average- fibre diameter $\mu$	'Size factor'
871	Auto-graft	Central	8,236	—	586	9.4	—	4.25	6,467
		Graft	6,528	105	176	2.7	30.0	3.52	4,691
		Peripheral	4,928	79	143	2.9	24.4	3.72	3,784
658	Homo-graft	Central	8,482	—	1319	16.4	—	4.85	8,890
		Graft	8,184	97	278	3.4	20.0	3.80	3,667
		Peripheral	7,733	91	201	2.6	14.5	3.76	4,703
602	Stored graft	Central	10,140	—	1724	17.0	—	4.95	9,382
		Graft	14,360	142	402	2.8	23.3	3.90	7,843
		Peripheral	10,200	101	306	3.0	17.7	3.20	3,992
601	Alcohol-fixed graft	Central	8,260	—	1817	22.0	—	5.00	10,415
		Graft	5,980	72	24	0.4	1.3	2.48	1,678
		Peripheral	4,983	60	84	1.7	4.6	3.02	2,098

*Central stumps.* Above nerve grafts the central stumps showed the same sort of changes as occurred when the nerve was repaired by plasma junction. The total number of myelinated fibres present was of the same order as those present in normal nerves, although the number and percentage of large fibres was reduced. In one case (the autograft) the average fibre diameter fell as low as 4.25  $\mu$  (compare 5.37  $\mu$  in normal nerve). In the case of the alcohol-fixed graft little diminution occurred. There were 22 % of large fibres and the average fibre diameter was 5.00  $\mu$ . However, study of the fibres within and below this 'graft' showed small total numbers of fibres, very small percentages of large fibres, and small average fibre sizes. The small number of myelinated fibres within the graft compared with the number in the central stump indicates that many fibres must have undergone deviation and blockage during the process of innervation of the 'graft'. Sanders & Young [1942] have shown that reinnervation of alcohol-fixed grafts takes place during the replacement of these grafts by the host tissue, the new fibres growing out all the time against resistance. Thus alcohol-fixed grafts are a type in which (a) many fibres are deviated or otherwise impeded and do not present long stretches of fibre to



be filled up by outflow from the central stump, (b) reconstitution is likely to be incomplete, in view of the unfavourable environment in which the new fibres find themselves, the majority of fibres not having increased in diameter beyond a certain small value. Thus it is not surprising to find that the diameters of the fibres in the central stump remains relatively high, although smaller than that of normal nerve.

Table 6 also shows that the fibres above stored and fresh homografts do not undergo as great a diminution as above an autograft. It has been shown above that alcohol-fixed grafts, innervated during replacement, offer a severe obstacle to new fibres. Similarly, Sanders & Young [1942] showed that stored homografts, in the early stages of reinnervation, contain many macrophages which may impede the outgrowth of new fibres, while fresh homografts contain many lymphocytes in addition to macrophages, which may have a similar effect. Autografts, behaving approximately like an ordinary peripheral stump, offer the least resistance to outflow. The frequency-distribution histograms of the size classes of the fibres found above these grafts are given in Fig. 10. In all cases there was a separate group of large fibres, but the mean of the group was at a lower diameter than in the case of normal nerve.

*Grafts.* Within the autograft, 200 days after its insertion, there were 6528 medullated nerve fibres, an increase of 5% over the number of fibres in the central stump. This increase is probably well within the limits of the experimental error, and cannot be regarded as significant. Thus the autograft at 200 days probably contained about the same number of fibres as in the central stump (see Table 6). The stored graft contained 14,360 fibres, an increase of 42% over the number of fibres seen in the central stump. This corresponds with the results seen by the many workers [see Aird & Naffziger, 1939, for summary] who have seen more fibres in the peripheral than in the central stump after suturing the central stump of a small nerve into the peripheral stump provided by resecting a nerve of larger diameter. Presumably the presence of a relatively large number of available pathways allows a greater number of the branches of the fibres of the central stump to survive and medullate. The present grafts were taken from the tibial, which has a larger diameter than the peroneal nerve in the same animal. The stored homograft used was much larger than its host nerve, being from a very large animal into a smaller host. In all the other cases the graft donor was the smaller animal and there was therefore little difference between the size of the graft and the host peroneal nerve which received it. Thus there was more discrepancy in the sizes of the host peroneal nerve and the graft it received in the case of the stored homograft than in the others. Moreover, the undesirable cell reactions which take place in the early stages of the reinnervation of fresh but not of stored homografts [Sanders & Young, 1942] may have caused fewer fibres to enter the fresh graft.

The fourth graft, made with an alcohol-fixed piece of nerve, contained 5980 fibres, 72% of the number present in its central stump (see Table 6).

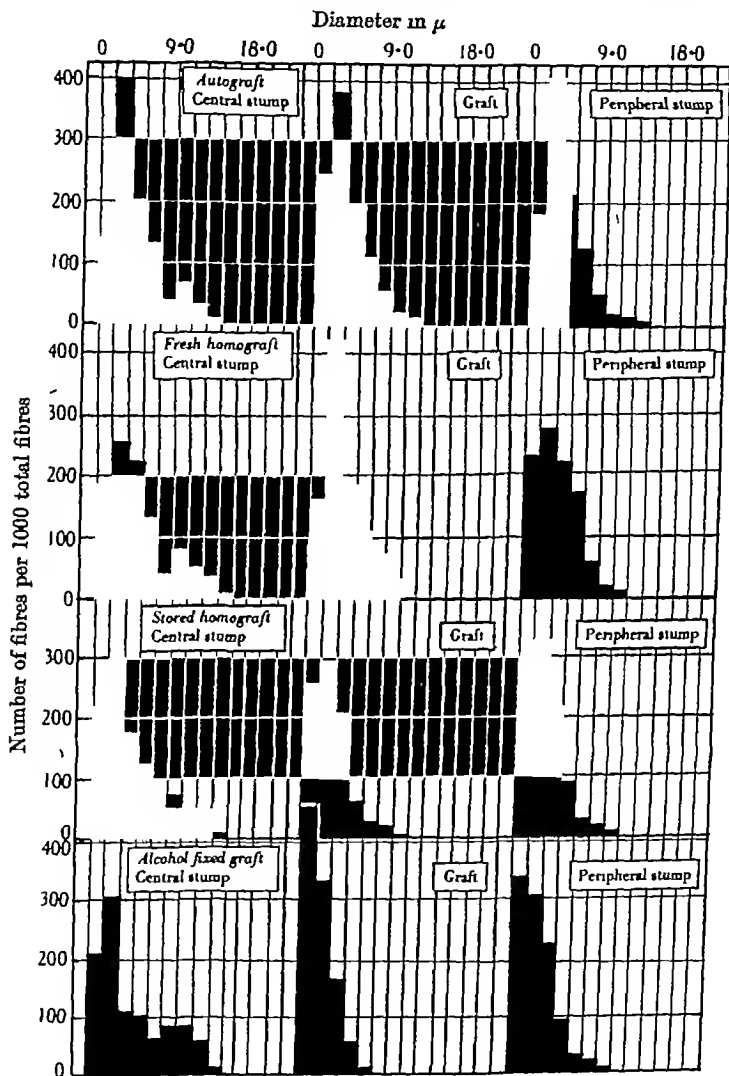


Fig. 10. Histograms of the fibre size spectrum (1) 15 mm. above the upper junction, (2) within the graft, (3) 10 mm. below the lower junction in the peripheral stump, 200 days after lesion and repair of the nerve by various nerve grafts.

The percentages of fibres larger than  $8\mu$  and the average fibre sizes for these four grafts are also given in Table 6. In the autograft 2.7% of the fibres were larger than  $8\mu$  in diameter, in the fresh homograft 3.4%, and in the stored graft 2.8%. These percentages are of the same order as those found in the

peripheral stumps 200 days after suture or grafting. By contrast only 0.4% of the fibres in the alcohol-fixed nerve reached a diameter greater than  $8\mu$ . In this case there were fewer large fibres in the graft than in the peripheral stump of the same nerve (see Table 6).

The 'average-fibre' diameter and size factors show a similar distribution. In the autograft, the fresh homograft and the stored graft they were respectively 3.52, 3.8 and  $3.9\mu$  and 4691, 3667, and 7843, the same sorts of value as found in the peripheral stump. The alcohol-fixed graft had an average fibre size of  $2.48\mu$ , and a size factor of 1678, smaller even than in the peripheral stump below a crush at 60 days.

Fig. 10 shows the fibre-size distribution histograms of these nerve grafts. Just as in the peripheral stumps (see below, p. 512) no separate group of fibres of large diameter was seen. All the grafts except the alcohol-fixed graft showed a large peak in the  $1.8-3.6\mu$  group, and thereafter the numbers of fibres in the successive size classes gradually declined. A few fibres of larger size than in a fresh homograft were found in an autograft and in a stored graft, but only a very small number. The peak in the distribution in the alcohol-fixed graft was in the  $1-1.8\mu$  group, and thereafter the diagram declined very steeply. There were no fibres of considerable diameter.

These results show a striking difference between the degree of reconstitution of the nerve shown by *incorporated grafts*, that is, grafts whose tissues remain and are incorporated into those of the host nerve [see Gutmann & Sanders, 1943], and *replacement grafts*, that is, grafts which are replaced by the tissues of the host, the replacing tissues forming a basis for the construction of a new nerve. In incorporated grafts 200 days after insertion the degree of reconstitution was very little different from that of a peripheral stump after end-to-end plasma junction. In a replacement graft the reconstitution was much inferior, the fibres being fewer in number and smaller in diameter. Moreover, within the 'graft' (i.e. the tissues replacing the graft) the fibres were smaller and less well medullated than in the peripheral stump at a lower level.

*Peripheral stumps.* The stump below the autograft contained 4928 fibres, 79% of the number of fibres in the corresponding central stump; i.e. fewer than in the graft above it. Below the fresh homograft there were 7733 fibres, 91% of the fibre content of the central stump, and again fewer than in the graft above it. The stump below the stored graft contained 10,200 fibres, about the same number of fibres as there were in the central stump, but many fewer than in the graft above it where the fibre number had increased. By contrast, the alcohol-fixed graft's peripheral stump contained only 4983 fibres, 60% of the number of fibres in the central stump. The autograft, homograft and stored graft stumps contained respectively 2.9, 2.6 and 3% of fibres larger than  $8\mu$ , compared with 9.4, 16.4 and 17.0% of large fibres in the central stumps. The average fibre sizes in these peripheral stumps were 3.72, 3.76

The fourth graft, made with an alcohol-fixed piece of nerve, contained 5980 fibres, 72% of the number present in its central stump (see Table 6).

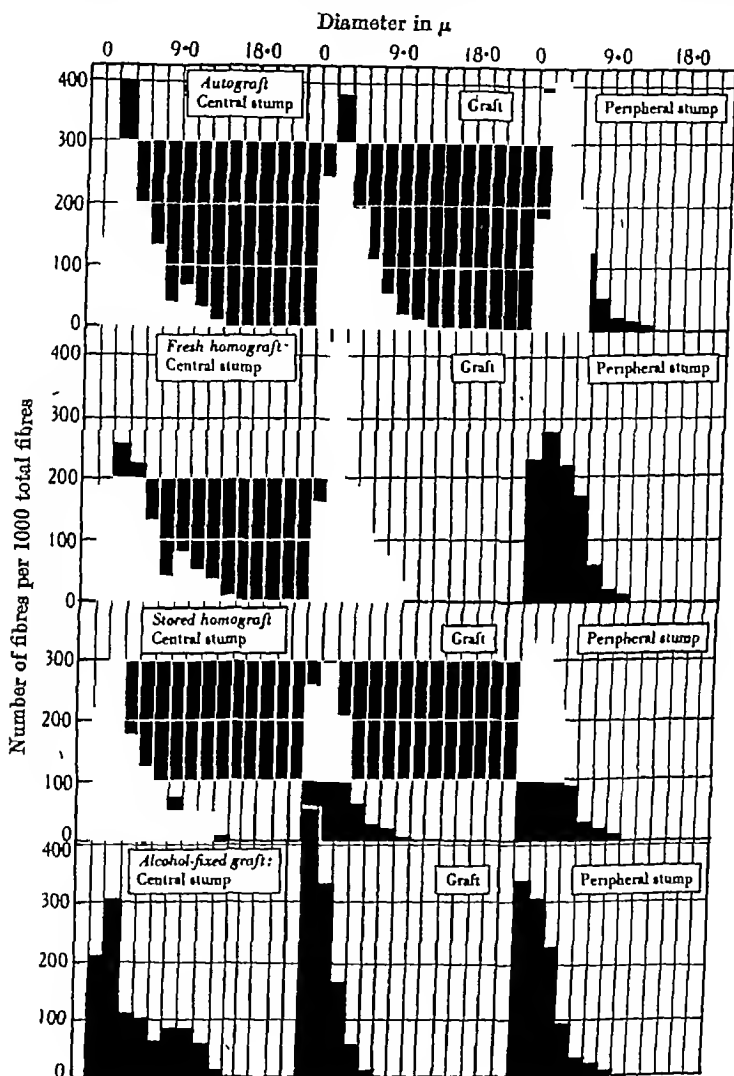


Fig. 10. Histograms of the fibre size spectrum (1) 15 mm. above the upper junction, (2) within the graft, (3) 10 mm. below the lower junction in the peripheral stump, 200 days after lesion and repair of the nerve by various nerve grafts.

The percentages of fibres larger than  $8\mu$  and the average fibre sizes for these four grafts are also given in Table 6. In the autograft 2.7% of the fibres were larger than  $8\mu$  in diameter, in the fresh homograft 3.4%, and in the stored graft 2.8%. These percentages are of the same order as those found in the

peripheral stumps 200 days after suture or grafting. By contrast only 0.4% of the fibres in the alcohol-fixed nerve reached a diameter greater than  $8\mu$ . In this case there were fewer large fibres in the graft than in the peripheral stump of the same nerve (see Table 6).

The 'average-fibre' diameter and size factors show a similar distribution. In the autograft, the fresh homograft and the stored graft they were respectively 3.52, 3.8 and  $3.9\mu$  and 4691, 3667, and 7843, the same sorts of value as found in the peripheral stump. The alcohol-fixed graft had an average fibre size of  $2.48\mu$ , and a size factor of 1678, smaller even than in the peripheral stump below a crush at 60 days.

Fig. 10 shows the fibre-size distribution histograms of these nerve grafts. Just as in the peripheral stumps (see below, p. 512) no separate group of fibres of large diameter was seen. All the grafts except the alcohol-fixed graft showed a large peak in the  $1.8-3.6\mu$  group, and thereafter the numbers of fibres in the successive size classes gradually declined. A few fibres of larger size than in a fresh homograft were found in an autograft and in a stored graft, but only a very small number. The peak in the distribution in the alcohol-fixed graft was in the  $1-1.8\mu$  group, and thereafter the diagram declined very steeply. There were no fibres of considerable diameter.

These results show a striking difference between the degree of reconstitution of the nerve shown by *incorporated grafts*, that is, grafts whose tissues remain and are incorporated into those of the host nerve [see Gutmann & Sanders, 1943], and *replacement grafts*, that is, grafts which are replaced by the tissues of the host, the replacing tissues forming a basis for the construction of a new nerve. In incorporated grafts 200 days after insertion the degree of reconstitution was very little different from that of a peripheral stump after end-to-end plasma junction. In a replacement graft the reconstitution was much inferior, the fibres being fewer in number and smaller in diameter. Moreover, within the 'graft' (i.e. the tissues replacing the graft) the fibres were smaller and less well medullated than in the peripheral stump at a lower level.

*Peripheral stumps.* The stump below the autograft contained 4928 fibres, 79% of the number of fibres in the corresponding central stump; i.e. fewer than in the graft above it. Below the fresh homograft there were 7733 fibres, 91% of the fibre content of the central stump, and again fewer than in the graft above it. The stump below the stored graft contained 10,200 fibres, about the same number of fibres as there were in the central stump, but many fewer than in the graft above it where the fibre number had increased. By contrast, the alcohol-fixed graft's peripheral stump contained only 4983 fibres, 60% of the number of fibres in the central stump. The autograft, homograft and stored graft stumps contained respectively 2.9, 2.6 and 3% of fibres larger than  $8\mu$ , compared with 9.4, 16.4 and 17.0% of large fibres in the central stumps. The average fibre sizes in these peripheral stumps were 3.72, 3.76

and  $3.20\mu$ , and the size factors 3784, 4703 and 3992, compared with 4.25, 4.85 and  $4.95\mu$ , and 6467, 8890 and 9382 in the corresponding central stumps. The stumps below the alcohol-fixed graft contained only 1.7 % of large fibres, and had an average-fibre size of  $3.02\mu$  and a size factor of 2098 as compared with 22 % of large fibres, an average-fibre size of  $5.0\mu$ , and a size factor of 10,415 in the central stump.

Fig. 10 also shows the fibre-size distribution histograms of the stumps taken below nerve grafts. In no case was there a separate large-fibre distribution, all cases except the alcohol-fixed graft showing a small-fibre peak at  $1.8-3.6\mu$ , and thereafter a gradual decline in the number of fibres in the successive size classes until the upper limit was reached. This upper limit was different in the case of the different grafts. In the stump below an autograft a larger fibre size was reached than in the case of the other grafts, but these large fibres were very few in number. In the alcohol-fixed graft the peripheral stump showed its small-fibre peak in the  $1-1.8\mu$  fibre group, the majority of its fibres being very small indeed. The upper limit of fibre size was the smallest recorded below any graft.

Compared with the peripheral stump below end-to-end suture made 200 days previously the stumps below autografts, fresh homografts and stored grafts at 200 days showed approximately the same percentage of large fibres and the same average-fibre size. The peripheral stump below an alcohol-fixed graft, on the other hand, showed a far smaller percentage of large fibres than below a suture, and a smaller average-fibre diameter. Moreover, the stump below an alcohol-fixed graft contained fewer fibres than the stumps below sutures or the other grafts at this time.

The recoveries shown by the autograft, fresh homograft, and stored homograft, although a little later in onset [see Gutmann & Sanders, 1943] were finally equal in degree to that following an end-to-end suture, that is, toe-spreading of considerable amplitude, but less than normal. In the alcohol-fixed graft, a much lower grade, just visible toe-spreading was the final degree attained.

Thus the fibres within and below the nerve grafts at 200 days do not show the degree of reconstitution found after simple crushes. Incorporated grafts are not markedly inferior to end-to-end sutures in this respect, but a replacement graft was much inferior. Comparison of the numbers of fibres within nerve grafts with those taken below in the peripheral stump (see Table 6) shows that in all cases there are fewer fibres in the periphery. The lower junction of nerve grafts may therefore constitute a point at which loss of fibres and also shunting can occur. However, Young [1942], has shown that in all probability the number of fibres in the peripheral stump which undergo maturation is determined by the number of Schwann tubes which it contains. Since the graft contains more tubes than the peripheral stump, a reduction

in the number of medullated fibres is to be expected in the peripheral stump, solely on account of the reduction in the number of available pathways. Moreover, the fact that there was no great difference in the degree of reconstitution of the nerve below grafts and sutures at 200 days, shows that shunting at the lower junction cannot have a great effect. Either shunting at a suture or the upper junction of a graft tends always to be maximal, so that shuffling of fibres at the second junction cannot disturb the pattern further, or else extensive shunting does not take place at the lower junction.

### DISCUSSION

From all these observations it is clear (a) that 300 days after the interruption of a nerve by simple localized crushing, the number, diameter and pattern of the fibres in the peripheral stump becomes fully restored to normal, (b) that 364 days after end-to-end suture, and 200 days after various grafts, few of the myelinated fibres in the peripheral stump have reached a diameter equal to that of the largest fibres in a normal nerve. Moreover, the bimodal frequency distribution seen in the histogram of fibre sizes in normal nerves and nerves which have recovered after crushing, has not been restored after these times in sutures or grafts. Thus reconstitution of the nerve is not complete after suture or grafting at a time much later than that at which functional recovery has reached the degree beyond which no further improvement appears to have taken place. Crush injuries also show this effect in the earlier stages. After crushing the maximum degree of recovery attained, as measured by the amplitude of toe-spreading [see Gutmann, 1943], is full normality, and this degree is reached within 60 days of operation when the crush is made 80 mm. from the muscle. Now at 60 days the nerve is far from normal, and contains no fibres of large diameter. Therefore a nerve which is not normal as far as the diameters of its constituent medullated fibres are concerned, can nevertheless function normally, at least to the degree necessary to produce at the end-plates a pattern of nervous impulses sufficiently like the normal to elicit the contractions of the muscles seen in a spreading reflex of normal amplitude. Quite a small degree of reconstitution at the level of the muscle, namely, about half the total number of medullated fibres and no fibres larger than  $8\mu$ , seems sufficient to initiate a spreading reflex of normal amplitude. Different functions, however, probably require different degrees of reconstitution. The spreading of the toes is a relatively crude function, brought about by the simultaneous contraction of a small number of muscles situated close together at about the same distance from the spinal cord. Although conduction rate depends on fibre diameter and the timing and succession of impulses is likely to be abnormal in incompletely reconstituted nerves, contraction of the muscles involved in toe-spreading can presumably occur without interference, as even when all the fibres are small the anatomical situation allows impulses to arrive

at the end-plates of the different muscles of the group without their time relations being greatly disturbed. Thus if sufficient muscle fibres have been reinnervated, apparently normal toe-spreading will be attainable, whatever the state of reconstitution of the nerve. Where a more complex movement is concerned, involving the precisely co-ordinated contraction of muscles at different distances from the cord, a greater degree of reconstitution will be required to restore function approaching normality. Thus in man, where the functions to be restored are generally more delicate than in the rabbit, reconstitution of regenerating nerves is of considerable importance.

After crushing, suture, or grafting, increase in the number and diameter of the fibres in the peripheral stump was always accompanied, at any rate to begin with, by a decrease in diameter of the fibres in the central stump, it being probable that the increase in diameter of the fibres in the periphery is due to an outflow from the cell bodies and the fibres in the central stump. Only after crushing was the initial decrease in diameter later reversed. As late as 364 days after suture the fibres above the lesion were smaller than in normal nerve.

It is not known what determines the final diameter reached by the fibres in the peripheral stump, or indeed what factor selects certain of the small initially non-medullated fibres and not others for subsequent thickening and medullation. Perhaps the simplest hypothesis is that the larger the fibre in the central stump, the greater the outflow, which would ensure that the fibres which were originally larger would reach the largest diameters. However, peripheral factors undoubtedly play a part, as exemplified by the different size of fibre reached at comparable times after different procedures (e.g. sutures and alcohol-fixed grafts). Young [1942] suggests that an important factor in determining the ultimate size of a fibre may be the diameter of the Schwann tube into which it penetrates. In an experiment in which the spinal nerve D 13 was sutured into the peripheral stump of the post-ganglionic trunk of the anterior mesenteric, the peripheral stump 100 days after operation was found to contain myelinated fibres, but these had a smaller diameter than the fibres in the peripheral stump 100 days after direct end-to-end suture of D 13. Moreover, Holmes & Young [1942] have found that after delayed suture of peripheral nerves the fibres in the peripheral stump are smaller than after immediate primary suture, and that the smaller diameter is due to shrinkage of the Schwann tubes of the peripheral stump during the long time that they are left uninnervated. Thus if reconstitution of the nerve plays a considerable part in the restoration of the more delicate functions in man, this is a strong argument against excessive delay of suture.

If the size of the peripheral Schwann tube plays a part in determining the final size of fibre, shunting of fibres will have an important effect, not only in directing fibres to the wrong end-organs, but in determining the final diameters



they will attain. Thus a large motor fibre shunted into the pathway occupied by a fibre of the B group, will be prevented by the small diameter of the tube from undergoing its full maturation. Conversely, a small fibre shunted into a large tube may not increase beyond its normal diameter, for if maturation of a fibre depends upon the outflow from the central stretch of fibre, the final size attained probably depends upon the size of the same fibre in the central stump. This effect has also been shown experimentally by Young [1942]. A nerve consisting almost entirely of non-myelinated fibres (post-ganglionic trunk of anterior mesenteric) was sutured into the peripheral stump of a nerve (D 13) which contained large medullated fibres, and therefore large tubes. Only a very few of the fibres in the peripheral stump became medullated and all remained of small diameter.

In the present experiments, after no suture or graft was toe-spreading of normal amplitude recovered, a state which was attained after every crush. Yet reconstitution was sometimes further advanced in the grafts and sutures (cf. Tables 3, 5 and 6) when these were compared with early crushes where functional recovery, but not reconstitution of the nerve, was complete. Shunting may account for this inferior recovery as explained on p. 507 as well as being responsible for the failure of the late stages of grafts and sutures to show as complete reconstitution as that seen in the final stages after crushing. Hence shunting adversely affects recovery in two ways: (a) by misdirecting fibres and thus reducing the number of end-organs reached by their own fibres, and (b) by preventing the full maturation of large fibres which get into small tubes. That this factor operates in man can be clearly seen. The statistics of both the last and the present war [Platt & Bristow, 1924; Seddon, personal communication] indicate that suture of certain nerves is more successful than others. For example, sutures of the radial nerve are followed by a much better recovery than sutures of the median or ulnar nerves. Now the radial nerve is primarily motor, while the sensory components of the median and ulnar nerves are much larger. Thus, whatever the degree of shunting taking place in a suture a misdirected motor fibre has a greater chance of finding a large motor tube in which it can make effective peripheral connexion and undergo proper maturation in the radial nerve than in either the median or the ulnar.

The existence of this control of fibre diameter by the size of Schwann tubes raises the question of the wisdom of using cable grafts made up of pieces of cutaneous sensory nerves to bridge large gaps in man. In rabbit nerves autografts have been markedly successful [Gutmann & Sanders, 1943], but the grafts have been short and taken from the tibial nerve, which is a mixed nerve and contains many large tubes. It is thus possible that some of the alleged failures of long autografts in man may be due to the inability of the new fibres within the small tubes of the cutaneous nerve bundles in

the graft to enlarge beyond a certain diameter and so conduct impulses with the proper time relations, to bring about a restoration of the more delicate functions.

However, many of the questions connected with reconstitution remain unsolved. Many fibres are found in a single Schwann tube in the early stages [see Holmes & Young, 1942], and it is not known what determines which of these shall be selected for maturation at the expense of the others. Moreover, it is not known what controls the initial deposition of myelin around a nerve fibre, or the final thickness it attains. An important factor is presumably axon diameter [Duncan, 1934; Schmitt & Bear, 1939]. All are factors which will affect the final distribution of fibre sizes seen in the nerves. At this stage it is enough to note that at very long periods after severance and suture of a nerve, or its repair by means of a nerve graft, the fibre diameter and pattern of fibres is not restored. Only after simple crushing does restoration take place, and this is not complete until long after the simpler functions have fully recovered.

#### SUMMARY

1. Counts and measurements of the myelinated nerve fibres in the peroneal nerve of the rabbit were made at standard levels: (a) in the normal nerve, (b) 50, 60, 70, 90, 100, 130, 200, 250 and 300 days after simple localized crushing, (c) 100, 200 and 364 days after severance and suture, (d) 200 days after various nerve grafts.

2. In the normal nerves there were 6000-9000 nerve fibres. In central stumps about the same number were found. After crushing a gradual increase in the number of medullated fibres in the peripheral stump took place, a number equal to that present in the central stump being found after 150-200 days. After suture there were always fewer fibres in the peripheral than in the central stump, the actual number present being larger the closer the apposition of the stumps at the suture line. After grafting there were always fewer fibres in the peripheral than in the central stump. After an alcohol-fixed graft only 60% of the central stump's number of fibres was found in the peripheral stump.

3. In the normal nerve there were nerve fibres varying in size from about 1 to  $20\mu$ , the majority being less than  $8\mu$  in diameter. The fibre diameters showed a frequency distribution which had two distinct peaks, one with a mean at 1.8-3.6 $\mu$ , and one at 12.6-14.4 $\mu$ .

4. After interruption of the nerve the myelinated fibres in the central stump 15 mm. above the lesion showed a reduction in diameter compared with normal nerve, as if they had become depleted by an outflow. In the case of crushes this decrease in diameter was progressive for at least 130 days, and thereafter the diameter gradually increased to the normal value. After suture or grafting no sign of the secondary increase was seen, the central stump fibres being smaller than normal even 364 days after suture.

5. After crushing, the fibres in the peripheral stump 10 mm. below the lesion showed a gradual increase in diameter until the normal diameter was attained after 250-300 days. In the early stages the frequency diagrams showed no separate population of large fibres. The secondary peak in the diagram due to this population first appeared definitely at 200 days, at a smaller size than normally, and reached its normal value by 250 days. At lower levels in the peripheral stump the restoration of the pattern of fibre sizes in the nerve was not complete until 300 days.

6. After suture or grafting the fibres in all peripheral stumps were on the average smaller than the fibres in the corresponding central stumps, even in a suture 364 days after operation. The fibre diameters had a frequency distribution in which there was no separate population of large fibres. Large fibres were present in all cases, but not in sufficient numbers or distributed in such a way as to show as a special peak in the fibre distribution histogram.

7. The largest fibres in the peripheral stumps after sutures and autografts were as large as those in the central stump, but there were many fewer of them. The large fibres in fresh and stored homografts were somewhat smaller. The fibres reached the least diameter in the tissues replacing alcohol-fixed grafts.

8. Only after crushing was the nerve fully reconstituted. Below two sutures at 364 days, and various nerve grafts at 200 days, the nerve was not restored to normality, as regards number, size, and pattern of fibres. The bearing of these results on functional recovery is discussed.

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# PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

27-28 March 1942

**A class experiment on urinary changes after exercise.** By  
M. GRACE EGGLETON. (*From the Pharmacology Department, University  
College, London*)

Opportunity arose during a special vacation course for a co-operative effort with the Biochemistry Department in organizing an experiment with the whole class of medical students. The results were so consistent that a brief description is given in the hope that it may prove useful to others.

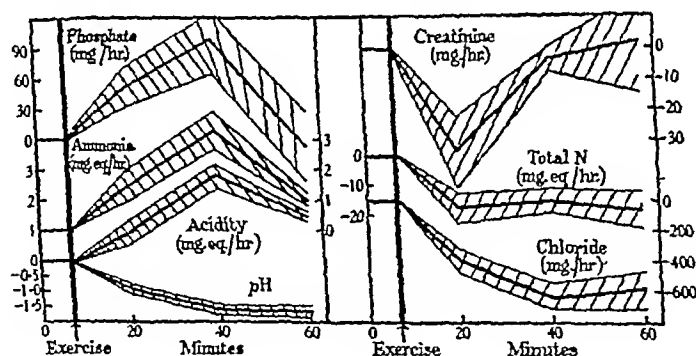


Fig. 1.

The class of sixty was divided into eight groups, from each of which one member volunteered as subject. The subjects reported for action in the morning, and the resulting urine samples were analysed by his group in the afternoon, each individual analysing one constituent in all samples.

Each subject drank 200 c.c. water every  $\frac{1}{4}$  hr., three times before and twice after the exercise. Two control samples of urine were collected (15-20 min. each) before the exercise, and three samples afterwards. The exercise consisted of running round a field, nearly  $\frac{1}{4}$  mile, in 50-60 sec.

This resulted in pronounced respiratory distress, greatly increased pulse rate, and considerable sweating. In seven out of the eight subjects, the urine flow decreased markedly in spite of the continued intake of water, falling from an average value of 6.6 c.c./min. before the exercise to 4.8, 3.0 and 1.0 c.c./min. in the three successive periods. The results obtained are shown in Fig. 1, the shaded areas indicating the standard deviation, which includes all errors, both chemical and physiological. The curves on the left require no further comment. Those on the right, taken in conjunction with the immediate decrease in urine flow, suggest a temporary renal vaso-constriction.

**Rayleigh interferometer arranged for rapid analysis of gas mixtures, or mixtures containing vapours.** By S. L. COWAN, H. G. EPSTEIN and S. F. SUFFOLK. (*From the Nuffield Department of Anaesthetics, University of Oxford*)

The interference refractometer was devised by Rayleigh [1896] and subsequently it has been improved by Haber & Löwe [1910], by Löwe [1912] and by the firms of Zeiss and of Hilger. It can be used for studying gaseous metabolism, or the progress of enzymic reactions [Hirsch, 1920, 1928, 1936]. At present ours is set up for the rapid determination of the concentration of ether vapour in mixtures. It is being used to study the performance of ether vaporizers for anaesthetic use, particularly the Oxford Vaporizer, and the absorption of ether vapour by substances such as charcoal. Once the instrument has been calibrated with mixtures of known composition, a single determination can be made within 1-2 min.

For producing the mixtures of known composition, a closed system of known volume, with a mercury manometer, can be evacuated. Gas mixtures are made by admitting known amounts of each component and mixing them thoroughly by a small circulating pump. Mixtures containing a known amount of vapour are prepared by inserting into the system and subsequently smashing an ampoule containing a weighed quantity of liquid: again, mixing is secured by the circulating pump. For calibration and for precise measurements monochromatic light, from a sodium discharge tube, is used. This by itself, however, would necessitate counting interference fringes. To eliminate that difficulty, an arrangement has been made whereby 'white' light, from a filament lamp, can be instantly substituted for sodium light. With white light, identification of the fringes

becomes easy, since all except the two which should be brought to the middle of the eyepiece field become widened and coloured at their edges. For measurements not requiring high precision white light can be used.

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# PROCEEDINGS

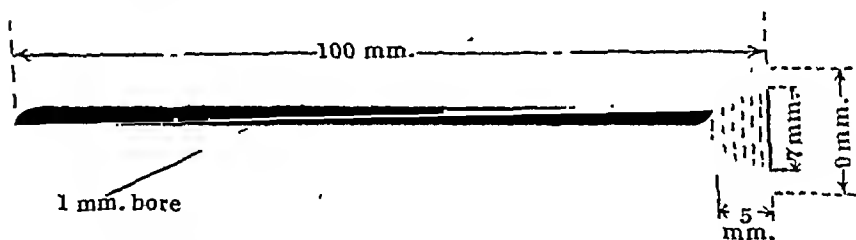
## OF THE

# PHYSIOLOGICAL SOCIETY

16 May 1942

Simple sealed haematocrit tube. By W. MEYERSTEIN.  
*Department of Physiology, University of Birmingham*

Ponder & Saslow [1930] described a haematocrit tube sealed at one end for use with small quantities of blood. This tube was difficult to fill and clean. Wintrobe [1929] used a similar tube of larger capacity requiring about 1 c.c. blood. The tube shown in the figure is made of thick wall



1 mm. bore capillary tubing. The open end is opened out into a cup-shaped hollow in which 1 or 2 drops of blood can be collected directly from a prick in the finger or lobe of the ear. One drop of a 0.1% heparin solution is placed in the cup and dried in an oven. After collecting the blood, a small glass plate is placed on the ground lip of the open end of the tube (to prevent evaporation) and held in place by a rubber cap. The tube is then spun in a centrifuge at 8000 r.p.m. for ten minutes. The blood displaces the air in the capillary and the levels are read and measured by placing the tube on squared paper.

For cleaning, a metal tube (hypodermic needle tubing, No. 22, 0.70 mm. outside diam.) sufficiently long to be inserted into the whole length of the capillary tubing is attached with suitable rubber connexions to a vacuum pump. The used haematocrit tube with needle tube in place is inverted in a vessel of distilled water and the pump

turned on. The blood is removed and replaced by water, which later can be removed and the tube dried by similar treatment with alcohol and ether.

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**Afferent impulses from the viscera.** By R. J. S. McDOWALL.  
*King's College, London*

In 1925, it was shown that the reactions of the pupil in a suitably chloralosed cat could be taken as the index of the setting up of afferent impulses from the viscera when they are stimulated, and that the method could be used to study the pathways concerned. The following alternative method, which is a modification of that of Langley, is, however, more convenient for demonstration to classes.

A cat is decerebrated under ether, and is allowed to rest for about two hours to allow excretion of the anaesthetic. The medulla is now put out of action by applying a clamp [McDowall, 1930] to the vertebral arteries and tying the carotids. In the majority of cats, this procedure produces, in about fifteen minutes, a spinal preparation devoid of shock, which is extremely sensitive to sensory stimulation. It may then be demonstrated that certain stimuli such as stretching the gut, or holding the heart firmly, cause marked limb movements, while others, such as cutting the gut, are without effect.

A successful result is only obtained if the decerebrate preparation is in good condition, as indicated by its rigidity, before occluding the cerebral arteries, and if care is taken not to over-ventilate the spinal preparation by the necessary artificial respiration.

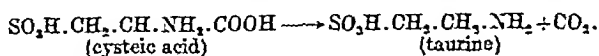
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**l(-)-Cystelic acid decarboxylase of dog's liver.** By H. BLASCHKO.  
*Physiological Laboratory, Cambridge*

Whereas the oxidative deamination of amino acids by enzymes has been relatively well studied, much less is known about decarboxylation of amino acids. Decarboxylases from mammalian tissue for tyrosine, his-

tidine and tryptophan have been described, but the rate of reaction is too slow to allow a study of these enzymes by manometric methods. This has so far been possible only in the case of *l*(-)-dopa decarboxylase [Holtz *et al.* 1938]. We have now found another enzyme of this group which lends itself to study by manometric methods. When cysteic acid is added to extracts from dog's liver in an atmosphere of nitrogen, carbon dioxide is formed. One molecule of  $\text{CO}_2$  is formed from one molecule of *l*(-)-cysteic acid and half a molecule of  $\text{CO}_2$  from *dl*-cysteic acid. The reaction catalysed must be the decarboxylation of *l*(-)-cysteic acid to taurine, thus:



It is likely that the new enzyme, *l*(-)-cysteic acid decarboxylase, takes part in the formation of taurine in the dog's liver. A scheme for the formation of taurine from cystine *via* cysteic acid was put forward by Friedmann [1903], and it is known that both cystine [Bergmann, 1904] and *l*(-)-cysteic acid [Virtue & Doster-Virtue, 1939] increase the output of taurocholic acid in the dog's bile. Moreover, in the rat's liver an enzyme which forms cysteic acid from cystine has been described [Medes, 1939].

The  $l(-)$ -cysteic acid decarboxylase shares with  $l(-)$ -dopa decarboxylase some characteristic properties. Both enzymes are reversibly inhibited by cyanide, are insensitive to octyl alcohol, and are stereospecific. These findings show that the mammalian amino acid decarboxylases form a well-defined class of enzymes with common properties. They catalyse reactions which do not constitute the principal pathway of amino acid breakdown, but which appear to serve specific functions in the elaboration of biologically important amines, such as taurine in the case of cysteic acid decarboxylase and possibly adrenaline or sympathin in the case of dopa decarboxylase [Blaschko, 1939].

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# PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

11 July 1942

**Serum choline esterase and muscular exercise.** By D. RICHTER and PHYLLIS G. CROFT (introduced by S. NEVIN). (*Central Pathological Laboratory (L.C.C.) and Mill Hill Emergency Hospital*)

The serum choline esterase activity generally remains very constant in normal individuals, and it is stated to be unaffected by muscular exercise in man [Hall & Lucas, 1937]. Contrary to this statement we have found a regular increase ranging from 15 to 51 % in the serum choline esterase activity as a result of vigorous muscular exercise. Following the initial rise there was a slow return to normal in the course of a few hours. The change was greater than could be accounted for as a haemoconcentration effect.

The effect of neuro-muscular activity on the serum choline esterase may be significant in relation to the changes in esterase activity that have been observed in certain clinical conditions. These include the *fall* in serum choline esterase activity (a) in catatonic stupor, (b) in extreme debility, and (c) in epilepsy and other conditions after treatment with narcotics as well as the *rise* in esterase activity, (d) in acute emotional states [Tod & Jones, 1937; Schütz, 1941; Richter & Lee, 1942].

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# PROCEEDINGS

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# PHYSIOLOGICAL SOCIETY

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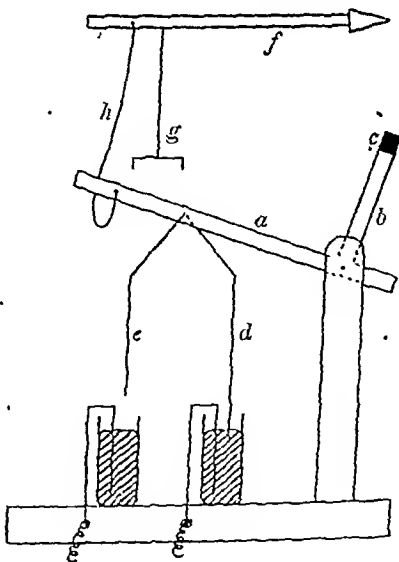
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# PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

3 October, 1942

**Continuous registration of pulmonary ventilation.** By D. H. SMYTH.  
(*Department of Physiology, University College, London*)

Continuous registration of the pulmonary ventilation is commonly made by recording the movements of a spirometer and using some device for its automatic periodic emptying. The following device, which is simple and effective, is easily set up. Expired air enters through one opening of the spirometer, another opening is connected with the inlet side of a respiration pump. The pump motor is in series with a relay and is started when the relay contact is made. The relay circuit contains, in series, the apparatus shown. A light lever (*a*) carries at its fulcrum a metal strip (*b*), weighted at its upper end (*c*). This weight causes the lever to fall to one or other side and remain there, until sufficient force is applied to move the weight to the other side of the vertical line. The arm of the lever carries a wire loop, the ends of which dip into mercury contacts. One limb (*d*) is sufficiently long that it always makes contact, the other (*e*) makes and breaks contact as the lever falls to one or other side, and thus makes and breaks the relay circuit. Attached to the writing pointer (*f*) of the spirometer is an arm (*g*) which, at a certain stage of filling, presses on the lever, pushes it down, makes the relay circuit and starts the pump,



which then empties the spirometer. At a certain stage of emptying, a thread (*h*) attached to the pointer pulls the lever to the other side, breaks the contact, and stops the pump, which remains stopped until the filling of the spirometer has again reached a certain level. A suitable relay with a resistance of about 3000 ohms will enable the whole apparatus to be run off the a.c. mains.



**Rheological properties of bovine cervical mucus.** By G. W. SCOTT BLAIR, A. T. COWIE and S. J. FOLLEY. (*National Institute for Research in Dairying, University of Reading*)

Viscous and elastic properties of bovine cervical secretions vary regularly during the oestrous cycle [Scott Blair, Folley, Malpress & Coppen, 1941] and the behaviour of mucus samples in a Scott Blair [1937] 'emptying tube' viscometer serves as a test for oestrus. For Newtonian (true) fluids under constant applied pressure,  $L^2 - l^2 = kt$ , where  $L$  = initial length of column and  $l$  its length after  $t$  sec. and  $k$  is a constant. The curves for cervical mucus are, however, usually non-linear, and their characteristics differ according as to whether the samples are from pregnant or non-pregnant animals [Scott Blair, Cowie & Coppen, 1942]. Utilizing only the lower parts of these curves, extrapolated intercepts on the vertical ( $L^2 - l^2$ ) axis are positive in oestrus, negative

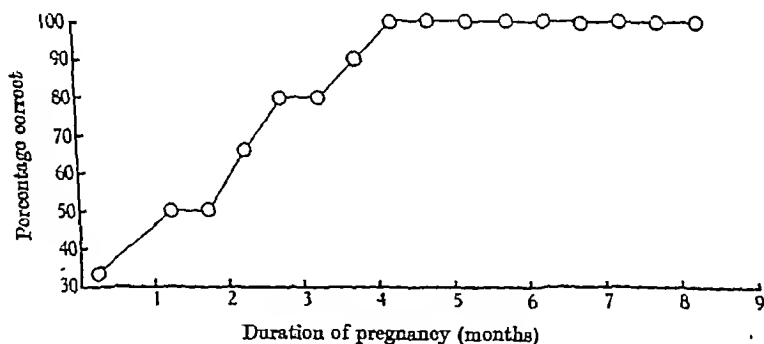


Fig. 1.

in pregnancy and almost zero in mid-cycle and very early pregnancy. In short, plasticity predominates in pregnancy and elasticity in the non-pregnant, and especially oestrous, condition.

Of fifty-nine non-pregnant cows tested, pregnancy diagnosis based on these findings was correct in fifty-six (95 %) cases. Unsatisfactory runs and samples, and a few suspected cases of anoestrus due to persistence of the corpus luteum, were not included. When replicates on animals studied over a number of oestrous cycles were included (155 samples), 96 % were correct.

The accuracy of diagnosis obtained with pregnant cows (eighty in all) is shown in Fig. 1 and is comparable with that obtainable by rectal palpation. The present test is purely empirical; improvement in theoretical knowledge of the rheological conditions involved should lead to increased accuracy.

Possible applications of these methods to studies of ovulation time and pregnancy diagnosis in women are being investigated by Prof. J. Chassar Moir and Dr M. Devenish Meares.

We are indebted to the Agricultural Research Council for a grant.

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# PROCEEDINGS

## OF THE

# PHYSIOLOGICAL SOCIETY

### 14 November 1942

**Absorption of volatile acids from the rumen of the sheep.** By R. A. MCANALLY and A. T. PHILLIPSON (introduced by J. BARCROFT). *From the Unit of Animal Physiology, Cambridge*

Volatile fatty acids are the chief non-gaseous product of bacterial breakdown of cellulose and other carbohydrates in the rumen. As such their subsequent fate is of major importance in nutrition of the ruminant. The fact that little if any volatile acid can be found in the abomasum suggests that it is absorbed before it reaches that organ. In order to test this theory a method for the distillation of volatile acid from blood has been developed.

Adult sheep, straight from pasture, were anaesthetized with nembatal, the abdomen was opened and blood was drawn from the veins draining the various compartments of the stomach, the small intestine and the caecum; samples were also taken from the jugular vein and carotid artery.

In all cases, while blood taken from veins draining the abomasum and small intestine and from the jugular vein and carotid artery contained little volatile acid (1-9, 1-6, 0-7, 1-9 c.c. of  $N/100$  acid per 100 c.c. of blood respectively), blood taken from veins draining the rumen contained notably larger quantities (17-71 c.c. of  $N/100$  per 100 c.c. of blood). The blood of tributary veins of the rumen did not all carry equal amounts of volatile acid; those draining the lower wall contained more than those draining the upper wall which, under the conditions of the experiment, was to a large extent separated from the ingesta by gas. The concentration of volatile acid in blood from the reticulum was of the same order as in the blood coming from the rumen, while blood from the omasum usually contained less volatile acid. Fermentation apparently also takes place in the caecum for significant quantities (11-20 c.c.  $N/100$  acids %) of the acids were present in the blood draining from that organ.

The rate of distillation under standard conditions indicates that acetic is the main volatile acid of venous blood from the rumen.

Two lambs were provided each with two rumen fistulae so that the rumen could be emptied. Blood from the empty rumen or the rumen filled with water contained no volatile acid; the introduction of a solution of sodium acetate caused an immediate increase in the concentration of volatile acid in the blood from the rumen; an equimolecular solution of sodium propionate caused a

somewhat smaller increase while an equimolecular solution of sodium butyrate caused no significant increase. This observation suggests that the rate of absorption depends on the size of the molecule.

Measurements of the outflow of blood from the posterior vein of the rumen together with the fraction of the surface of the rumen and reticulum it drains indicate that from 2 to 4 grams of acetic acid are absorbed from the rumen in an hour.

Thus absorption of products of digestion of cellulose occurs in the stomach and is not delayed until the small intestine is reached.

### **Absorption of sodium ortho-iodo hippurate from the rumen of lambs.**

By R. A. McANALLY and A. T. PHILLIPSON (introduced by J. BARCROFT).

*From the Unit of Animal Physiology, Cambridge*

In a recent paper [Phillipson & McAnally, 1942], it was suggested that absorption occurred from the rumen of the sheep, as it was otherwise impossible to account for the disappearance of the lower fatty acids formed as a result of the fermentation of carbohydrates. Evidence in favour of this supposition was discussed. The present experiments were performed to find out whether absorption through the stratified epithelial lining of the rumen, reticulum and omasum is possible.

Six lambs were used, four as experimental animals and two as controls. The abdomen was opened under nembutal anaesthesia and a ligature was passed between the omasum and abomasum excluding the epiploic vessels. The radio-opaque salt, sodium ortho-iodo hippurate, was administered at the rate of approximately 2 g./kg. body weight by stomach tube leading to the rumen. Radiographs were taken at intervals before and after dosing. The urethra was ligated in one case to prevent escape of urine, but this was found to be unnecessary. The increase in opacity of the bladder contents indicated clearly that the salt was absorbed from the rumen. The rate of absorption was slow; a distinct shadow of the bladder was visible 1 hr. after dosing, but was not dense until 4-5 hr. after dosing. In control experiments the lambs were given water to show the faint shadow of normal urine.

The position and security of the ligature was tested at the end of each experiment, and the presence of organic iodine in the urine was demonstrated chemically. It is concluded that absorption can occur through the stratified epithelium of the first three compartments of the ruminant stomach.

We thank Glaxo Laboratories for the supply of the opaque drug and Mr J. A. F. Fozzard, Radiographer of the Anatomy School, for his active assistance.

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**The effect of haemorrhage on blood fat.** By H. C. STEWART.\*

*From the Department of Physiology, St Mary's Hospital, W. 2*

The blood-fat (triglyceride) level in the rabbit remains constant at about 120-150 mg./100 c.c. while on its customary diet. For this reason it is a convenient animal in which to study alterations in blood fat from endogenous sources.

When the red-cell count has been reduced to the 2 million mark or below, after repeated daily bleedings of 20-25 c.c., the serum appears quite milky. This is in agreement with Boggs & Morris [1909]. Similar results were obtained when an equivalent degree of anaemia was produced with phenylhydrazine.

Blood fat was estimated by a gravimetric modification of Bloor's method [Elkes, Frazer & Stewart, 1939], and for frequent observations by the lipomicrograph technique [Frazer & Stewart, 1939]. The degree of lipaemia produced follows more closely the bone-marrow activity, as expressed by the reticulocyte increase, than it does the depression of the red cell count.

\* Sir Halley Stewart Research Fellow.

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